

**REMARKS**

**I. Claim Status**

Claims 1-43 are pending. Claims 4, 6, 10, 11, and 18-43 have been withdrawn. Claims 1-3, 5, 7-9, and 12-17 have been rejected.

Applicant cancels claims 2 and 10-12.

Applicant amends claim 1 to recite a conjugate molecule consisting of an oligo- or polysaccharide selected from the group consisting of {AB(E)CD}<sub>n</sub>, wherein A is an  $\alpha$ LRhap-(1,2) residue, B is an  $\alpha$ LRhap-(1,3) residue, C is an  $\alpha$ LRhap-(1,3) residue, E is an  $\alpha$ DGlc<sub>p</sub>-(1,4) residue, D is a  $\beta$ DGlcNAc<sub>p</sub>-(1,2) residue, E is branched to C, and wherein n is an integer selected from 2, 3, covalently bound to a carrier. This amendment is supported for example in original claim 12, and in Figure 4 of the specification, which shows the synthesis of the AB(E)CD pentasaccharide 102.

Claim 14 is amended by deleting the wording "such as a decasaccharide or a pentadecasaccharide". Applicant presents claim 44, which recites "[t]he composition of claim 14, wherein said oligo- or polysaccharide is a decasaccharide or a pentadecasaccharide". Claim 44 does not introduce new subject matter and is supported by original claim 14.

Applicant presents the current claim amendments in order to expedite the allowance of the application. However, Applicant wishes to reserve the right to pursue deleted subject matter in one or more continuation applications.

Applicant respectfully requests consideration of claims 1, 3, 5, 7-9, 13-17 and 44.

## II. Rejections Under 35 U.S.C. § 112

Claims 15-16 were rejected under 35 U.S.C. § 112, first paragraph, because the specification does not reasonably provide enablement for compositions which afford “protection”, and given the lack of guidance, and the unpredictable nature of the invention, one of skill in the art would be forced into excessive experimentation in order to practice the instantly claimed invention. Office Action at 2-3.

Applicant respectfully traverses. However, Applicant amends claim 15 by deleting the following pathogens: *S. flexneri* serotype 1b, 3a and 6, and *S. species* such as *S. dysenteriae* and *S. sonnei*. Thus, claim 15 recites “[t]he composition of claim 14, comprising an immunogen which affords protection against pathogens responsible for diarrhoeal disease in humans”. Support for this claim is found in the specification: “[p]rotection as assessed by reduction of the bacterial load, was observed with the penta, deca and pentadecasaccharides conjugates...”. See specification at p. 143, l. 18-21, and Figure 34; emphasis added.

Claims 14-15 were rejected because the phrase “such as” renders the claim indefinite. Office Action at 3. Claims 14-15 have been amended to delete the phrase “such as”.

Claim 3 was rejected under 35 U.S.C. § 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention. Further, the Office contends the claim is vague in the use of the phrase “derivative”. Office Action at 3-4.

Claim 3 is amended to recite that the derivative “is recognized by T-cells and is able to induce an antibody response”. This amendment is supported in the specification at p. 8, l. 10-11.

Applicant submits that all rejections under 35 U.S.C. § 112 are obviated by the currently amended claims and respectfully requests the rejections are withdrawn.

### **III. Rejections Under 35 U.S.C. § 102(b) and 103(a)**

Claims 1-3, 5, 7-9, and 12-17 were rejected under 35 U.S.C. § 102(b) as allegedly being anticipated by or, in the alternative, under 35 U.S.C. § 103(a) as allegedly obvious over Polotsky et al. (Infection and Immunity 62: 210-214, 1994). Office Action at 5.

Applicant respectfully traverses. According to the MPEP, a claim is anticipated “only if each and every element as set forth in the claims is found, expressly or inherently described, in a single prior art reference”. See MPEP § 2131 (quoting *Verdegaal Bros. v. Union Oil Co. of California*, 814 F.2d 628, 631 (Fed. Cir. 1987)). Applicant submits that Polotsky et al. does not describe each element of the amended claims.

It is known that the immune response against *Shigella* infection is serotype specific and the O-specific polysaccharide (O-SP or O-antigen) is the major protective antigen that defines *Shigella*'s species and serotypes. The O-antigen consists of heteropolysaccharide chains of lipopolysaccharide (LPS), which are made up of repeated saccharide units (RU). For example, the tetrasaccharide component of *Shigella flexneri* (*S. flexneri*) is ABCD, the *S. flexneri* type 2a specific unit is AB(E)CD, in

which E is branched to C, and the *S. flexneri* type 5a specific unit is A(E)BCD, in which E is branched to B.

Conventional polysaccharides are obtained by cutting the natural O-antigen by acidic hydrolysis, which cleaves the glycosidic link to lipid A. The fragments comprise between 15,000 and 20,000 RU. Antigens obtained from the natural O-antigen of LPS have a molecular weight of between 12,000 and 16,000 daltons (D). Hydrolysis of a natural LPS can only give a mixture of very low to very high molecular weight structures. Methods are not available to control the molecular weight distribution of such molecules apart from chemical synthesis, as described in the present application.

Applicant's claims are directed to a conjugate consisting of a deca- or pentadecasaccharide of formula:  $\{AB(E)CD\}_n$ , wherein  $n$  is 2 or 3, and wherein the deca- or pentadecasaccharide is covalently bound to a carrier. These synthetic conjugates are homogenous with regard to their structure and molecular weight. Thus, considering that the molecular weight of each saccharide unit (A to D) is approximately 200 D, the molecular weight of Applicant's deca- or pentadecasaccharide is approximately 2000 D ( $200 \times 5 \times 2$ ), or 3000 D ( $200 \times 5 \times 3$ ), respectively.

Polotsky et al. discloses conjugates of the *S. flexneri* type 2a LPS, wherein LPS has been detoxified with acetic acid (O-SP) or with hydrazine (DeAPLS). Polotsky et al. shows that O-SP has a molecular weight of 17,000 D, i.e. a peak corresponding to an  $M_r$  of approximately 17,000 D by high-pressure liquid chromatography (HPLC), and DeALPS has a peak with an  $M_r$  of 30,000 D and a minor peak with an  $M_r$  of 10,000 D. See Polotsky et al. at p. 212, left column. These conjugates are heterogenous because they have been obtained by hydrolysis. *Id.* Notably, the O-SP and DeALPS are

mixtures which contain the tetrasaccharide component of *S. flexneri* type 2a LPS. *Id.* at p. 210, left column.

Polotsky et al. does not describe the presence of conjugates consisting of a deca- or pentadecasaccharide, as recited in Applicant's amended claims. Nor does Polotsky et al. describe conjugates with molecular weights similar to conjugates consisting of a deca- or pentadecasaccharide. Thus, Polotsky et al. does not describe each element of Applicant's amended claims. Therefore, Applicant respectfully requests the rejection under 35 U.S.C. § 102(b) is withdrawn.

Applicant also submits that the claims are not obvious over Polotsky et al. because the results obtained with Applicant's claimed conjugates could not have been predicted by one of skill in the art in view of Polotsky's results. This is supported by the fact that the development of a vaccine for *S. flexneri* has been a high priority and remains a problem to be solved by those in the field (specification at p. 1, l. 35-38 through p. 2, l. 1-9), and Polotsky's approach to a vaccine, which was reported in 1994, had a number of problems which are solved by Applicant's synthetic conjugates.

Polotsky et al. demonstrated the feasibility of using a natural O-antigen preparation conjugated to a protein/peptide carrier to elicit serum antibodies against *S. flexneri*. However, there are multiple problems with Polotsky's approach to a vaccine, including that the composition cannot be standardized and the immunogenicity is not reproducible due to the heterogeneity of the natural O-antigen preparations. In contrast, Applicant focused on the development of a "well-defined neoglycoconjugate as an alternative to polysaccharide protein conjugate vaccines targeting infections caused by *S. flexneri* serotype 2a". *Id.* at p. 4, paragraph 2. As a result, Applicant solved the

problem of providing a synthetic glycoconjugate for the preparation of a vaccine against *S. Flexneri* infections.

It was not expected that Applicant's claimed conjugates would work to solve the problem of a *S. flexneri* vaccine in view of Polotsky et al. In fact, Applicant showed that mice immunized with detoxified LPS 2a conjugates were not protected against a challenge of *S. flexneri* infection. *Id.* at Figure 34. With regard to the immunogenicity of the vaccine, Applicant's data showed that the claimed conjugates were able to induce high anti-LPS 2a antibody titers in all immunized mice and that these antibodies bind with high affinity to the claimed glycoconjugate. *Id.* at Table H to K, and Figure 34. In contrast, mice immunized with conjugates consisting of a tetrasaccharide or hexasaccharide did not produce anti-LPS 2a antibodies, despite having an anti-oligosaccharide response. *Id.* at Table J. Mice that were immunized with detoxified LPS conjugates produced only low levels of anti-LPS 2a antibodies. *Id.* at Table H. The results of the protection studies correlate with those of the anti-LPS 2a antibody response, wherein most of the mice immunized with pentadecasaccharide and decasaccharide conjugates were protected against *S. flexneri* challenge, whereas mice immunized with tetrasaccharide, hexasaccharide, or the detoxified LPS 2a conjugates were not protected against infection. *Id.* at Figure 34. Applicant's data demonstrate that the antibody response was improved, and ultimately protection from infection was achieved with the length of the synthetic oligosaccharide, which was unexpected in view of Polotsky et al's results with detoxified LPS conjugates.

Applicant's results demonstrating the superior immunogenic and protective effect of the claimed conjugates have been further confirmed in peer-reviewed articles, which

have been published since the filing date of the present application. These references include Phalipon et al. (Journal of Immunology 176: 1686-1694, 2006), Phalipon et al. (Microbes and Infection 10: 1057-1062, 2008), and Phalipon et al. (Journal of Immunology 182: 2241-2247, 2009). Applicant has provided the above references for the Examiner's consideration.

Applicant respectfully requests the rejection under 35 U.S.C § 103 (a) is withdrawn in view of the unexpected results obtained with the claimed conjugates, which would not have been obvious to one of skill in the art in view of Polotsky et al.

#### **IV. Conclusion**

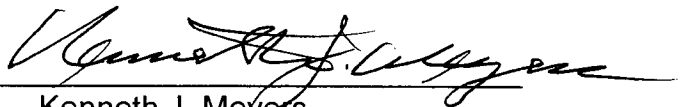
In view of the foregoing amendments and remarks, Applicant respectfully requests reconsideration and reexamination of this application and the timely allowance of the pending claims.

Please grant any extensions of time required to enter this response and charge any additional required fees to our deposit account 06-0916.

Respectfully submitted,

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# Characterization of Functional Oligosaccharide Mimics of the *Shigella flexneri* Serotype 2a O-Antigen: Implications for the Development of a Chemically Defined Glycoconjugate Vaccine<sup>1</sup>

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Protection against reinfection with noncapsulated Gram-negative bacteria, such as *Shigella*, an enteroinvasive bacterium responsible for bacillary dysentery, is mainly achieved by Abs specific for the O-Ag, the polysaccharide part of the LPS, the major bacterial surface Ag. The use of chemically defined glycoconjugates encompassing oligosaccharides mimicking the protective determinants carried by the O-Ag, thus expected to induce an efficient anti-LPS Ab response, has been considered an alternative to detoxified LPS-protein conjugate vaccines. The aim of this study was to identify such functional oligosaccharide mimics of the *S. flexneri* serotype 2a O-Ag. Using protective murine mAbs specific for *S. flexneri* serotype 2a and synthetic oligosaccharides designed to analyze the contribution of each sugar residue of the branched pentasaccharide repeating unit of the O-Ag, we demonstrated that the O-Ag exhibited an immunodominant serotype-specific determinant. We also showed that elongating the oligosaccharide sequence improved Ab recognition. From these antigenicity data, selected synthetic oligosaccharides were assessed for their potential to mimic the O-Ag by analyzing their immunogenicity in mice when coupled to tetanus toxoid via single point attachment. Our results demonstrated that induction of an efficient serotype 2a-specific anti-O-Ag Ab response was dependent on the length of the oligosaccharide sequence. A pentadecasaccharide representing three biological repeating units was identified as a potential candidate for further development of a chemically defined glycoconjugate vaccine against *S. flexneri* 2a infection. *The Journal of Immunology*, 2006, 176: 1686–1694.

*Shigella* infection represents about one-third of the total deaths due to diarrheal diseases (1). *Shigella flexneri* is responsible for the endemic form of shigellosis, a dysenteric syndrome characterized by a spectrum of symptoms varying from watery diarrhea to severe dysentery. These symptoms largely reflect bacterial invasion into the colonic and rectal mucosa that results in an acute inflammation responsible for massive tissue destruction (2).

Upon natural infection or following vaccine trials, as well as during experimental infection, the Ab-mediated protection has been shown to be serotype specific, pointing to the LPS as the major protective Ag (2). *Shigella* serotypes are defined by the

structure of the oligosaccharide repeating unit (RU)<sup>3</sup> that forms the O-Ag, the polysaccharide part of LPS (3). For the predominant *S. flexneri* serotype 2a, the biological RU is the branched pentasaccharide shown in Fig. 1. It bears a linear tetrasaccharide backbone made of three L-rhamnose residues, A, B, and C, and a N-acetyl-D-glucosamine residue D, that is common to all *S. flexneri* except serotype 6 and represents the RU of *S. flexneri* serotype Y. The RU of serotype 2a is characterized by the presence of the  $\alpha$ -D-glucose residue E, branched at position 4 of rhamnose C on the linear tetrasaccharide backbone.

So far, widespread use of a safe and effective vaccine appears to be the only strategy to control *Shigella* infection. In addition to orally administered live, attenuated vaccine strains (2), glycoconjugate vaccines using detoxified LPS as the main protective Ag coupled to a protein carrier have recently been developed, essentially as an extension of the successful human vaccination with bacterial capsular polysaccharide-protein conjugate vaccines. Encouraging results were obtained with a detoxified *Shigella sonnei* LPS-based conjugate vaccine administered parenterally, showing protection in ~75% of the vaccines during a *S. sonnei* outbreak (4). However, glycoconjugate vaccines based on the use of detoxified LPS present some drawbacks such as the need for accurate control of the detoxification step and also the potential loss of immunogenicity upon coupling to the protein carrier. In addition, to fulfill the requirements of regulatory agencies for always better-defined molecules to be used in humans, an alternative strategy has been proposed. It is based on the conjugation onto appropriate

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<sup>3</sup> Abbreviations used in this paper: RU, repeating unit; mIgG, mouse IgG; TT, tetanus toxoid; i.n., intranasal; PMN, polymorphonuclear; CP, capsular polysaccharide.

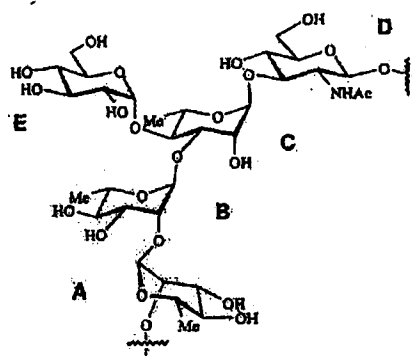


FIGURE 1. Structure of the repeating unit of the O-Ag of *S. flexneri* serotype 2a.



carriers of synthetic oligosaccharides mimicking determinants which are targets for protective Abs, with the hope that they induce an anti-LPS Ab response. Recent data have confirmed the assumption that short fragments of polysaccharides may mimic the native Ag. Indeed, semisynthetic glycoconjugates incorporating fragments of bacterial polysaccharide Ags were shown to be highly immunogenic in mice (5–7). The “proof of concept” was recently provided in humans with the efficacy of such a semisynthetic glycoconjugate in protecting against *Haemophilus influenzae* b infection (8).

The aim of this study was to identify functional mimics of the *S. flexneri* serotype 2a O-Ag. We chose to identify the protective serotype 2a-specific determinants by performing an extensive study using synthetic oligosaccharides to define the contribution of each sugar residue in the recognition by protective serotype 2a-specific mouse mAb of the G isotype (mIgG). First of all, five mIgG specific for *S. flexneri* serotype 2a and representative of each IgG subclass were selected, and their protective efficacy was assessed in a murine model of infection (9, 10). Then, available mono-, di-, tri-, tetra-, and pentasaccharides representative of the RU, as well as longer sequences, were tested for their recognition by these five mIgG using inhibition ELISA to define an IC<sub>50</sub>. A set of oligosaccharides was next selected based on these antigenicity data. Their potential as accurate mimics of serotype 2a O-Ag, i.e., their ability to induce anti-O-Ag Abs, was assessed in mice upon immunization with the corresponding tetanus toxoid (TT) glycoconjugates. We, finally, succeeded in identifying a potential candidate for further development of a chemically defined glycoconjugate vaccine to *S. flexneri* 2a.

## Materials and Methods

### Bacterial strains

M90T, an invasive isolate of *S. flexneri* serotype 5a, and 454, an invasive isolate of *S. flexneri* serotype 2a, were the virulent strains of reference. For intranasal (i.n.) infection, bacteria were routinely grown on Luria-Bertani agar plates at 37°C. They were recovered from plates and bacterial dilutions were performed in 0.9% NaCl with the consideration that, for an OD of 1 at 600 nm, the bacterial concentration was  $5 \times 10^8$  CFU/ml. Killed bacteria for i.p. immunizations were prepared from bacterial cultures at stationary phase, diluted to  $5 \times 10^8$  CFU/ml in 0.9% NaCl, and then incubated at 100°C for 1 h. They were then kept at –20°C in aliquots.

### Production and characterization of mAbs specific for *S. flexneri* serotype 2a LPS

BALB/c mice were immunized i.p. with  $10^7$  CFU of killed *S. flexneri* 5a or *S. flexneri* 2a bacteria three times at 3-wk intervals. Mice eliciting the

highest anti-LPS Ab response were given an i.v. booster injection 3 days before being sacrificed for splenic B cell fusion according to Kohler and Milstein (11). Hybridoma culture supernatants were screened for Ab production by ELISA using LPS purified from *S. flexneri* serotype X, Y, 5a, 5b, 2a, 2b, 1a, and 3a, respectively, as previously described (12, 13). Briefly, LPS purified according to Westphal and Jann (14) was used at a concentration of 5  $\mu$ g/ml in PBS. As secondary Abs, anti-mouse IgG-, IgM-, or IgA-alkaline phosphatase-labeled conjugate (Sigma-Aldrich) were used at a dilution of 1/5,000.

Only the hybridoma cells secreting mIgG reacting specifically with LPS homologous to the strain used for immunization, i.e., recognizing serotype-specific determinants on the LPS O-Ag were selected. Those selected, representative of the four murine IgG subclasses, were then cloned by limiting dilution, and injected i.p. into histocompatible mice for ascites production. mIgG were precipitated with 50% ammonium sulfate from ascites fluid, centrifuged, and dialyzed against PBS before being purified using ion-exchange chromatography as previously described (12, 13).

### mIgG sequence analysis

Total RNA was extracted from hybridoma cells by RNaxel kit (Eurobio). mRNA was converted into cDNA with a reverse transcriptase kit (Invitrogen Life Technologies) and used as template for PCR amplification using TaqDNA polymerase (Invitrogen Life Technologies) according to the manufacturer's protocol. The amplification was performed with the primer of corresponding isotype (IgG1, 5'-GCA AGG CTT ACT AGT TGA AGA TTT GGG CTC AAC TTT CTT GTC GAC-3'; IgG2a, 5'-GTT CTG ACT AGT GGG CAC TCT GGG CTC-3'; and IgG3, 5'-GGG GGT ACT AGT CTT GGG TAT TCT AGG CTC-3'). The following eight H chain variable region (V<sub>H</sub>) primers were also used: 5'-AG GTG CAG CTC GAG GAG TCA GGA CC-3'; 5'-GAG GTC CAG CTC GAG CAG TCT GGA CC-3'; 5'-CAG GTC CAA CTC GAG CAG CCT GGG GC-3'; 5'-GAG GTT CAG CTC GAG CAG TCT GGG GC-3'; 5'-GAG GTG AAG CTC GAG GAA TCT GGA GG-3'; 5'-GAG GTA AAG CTC GAG GAG TCT GGA GG-3'; 5'-GAA TGT CAG CTC GAG GAG TCT GGG GG-3'; and 5'-GAG GTT CAG CTC GAG CAG TCT GGA GC-3'. For the light chains, the primer sequences were: for the  $\kappa$ -chain, 5'-GCG CCG TCT AGA ATT AAC ACT CAT TCC TGT TGA A-3'; for the variable region (V<sub>L</sub>), 5'-CCA GTT CCG AGC TCG TTG TGA CTC AGG AAT CT-3'; 5'-CCA GTT CCG AGC TCG TGT TGA CGC AGC CGC CC-3'; 5'-TGG ATG GTG GGA AGA TG-3'; 5'-GAG AGC AGA AAT AAA CTC CC-3'; 5'-CCA GAT GTG AGC TCG TGA TGA CCC AGA CTC CA-3'; 5'-GAC CCC AGA AAA TCG GTT-3'; 5'-CCA GTT CCG AGC TCG TGA TGA CAC AGT CTC CA-3'; 5'-TTC CCA GGC TGT TGT GA-3'; 5'-GAG CTC GTG ATG ACA CAG TCT CCA-3'; and 5'-AAT TCT AAC TAG CTA GTC GCC-3'. Nucleic acid sequence determination was conducted by Genome Express using PCR products. Sequence analysis was performed with software packages from the Genetics Computer Group (Madison, WI), the GenBank (Los Alamos, NM), and European Molecular Biological Laboratory (Heidelberg, Germany) databases. For the determination of the gene families, analysis of the nucleotide sequences was performed with the international ImMunoGeneTics database (<http://imgt.cines.fr>) (15). The determined sequences have been submitted to the European Molecular Biological Laboratory nucleotide sequence database

(<http://www.ebi.ac.uk>) with the accession numbers from AJ 784030 to AJ784039.

### Protection experiments

Purified mIgG (20 or 2  $\mu$ g) were administered i.n. using a volume of 20  $\mu$ l to mice previously anesthetized via the i.m. route with 50  $\mu$ l of a mixture of 12.5% ketamine (Merial) and 12.5% acepromazine (Vetoquinol). Intranasal challenge was performed using  $10^8$  virulent bacteria in a volume of 20  $\mu$ l (10). Measurement of lung bacterial load was performed at 24 h after infection as follows. Mice were sacrificed by cervical dislocation and lungs were removed en bloc and ground in 10 ml of sterile PBS (Ultra Turrax T25 apparatus, Janke and Kunkel IKA Labortechnik). Dilutions were then plated on trypticase soy broth plates for CFU enumeration. The lung bacterial load in mice receiving the mIgG was compared with that of control mice receiving PBS. Each experiment was performed using 10 mice per group and repeated three times. All of the animal experiments were approved by the Institut Pasteur Animal Use Committee.

### Histopathological studies

Mice were anesthetized, their trachea catheterized, and 4% Formalin injected to fill the bronchoalveolar space. Lungs were then removed and fixed in 4% Formalin before being processed for histopathological studies. Ten-micrometer paraffin sections were stained with H&E or labeled with specific Abs and observed with a BX50 Olympus microscope (Olympus Optical, Europa).

### Synthetic oligosaccharides representative of *S. flexneri* 2a O-Ag

Oligosaccharides representative of fragments of the O-Ag of *S. flexneri* 2a were synthesized as their methyl glycoside to reproduce the ring and anomeric forms that the reducing residue adopts in the natural polysaccharide (see Table I). The serotype-specific oligosaccharides, thus bearing residue E, were obtained through multistep chemical synthesis as previously reported (16–19). The BEC disaccharide (16), which has a non-natural EC glycosidic linkage, was synthesized to probe the influence of such linkage on Ab recognition. Since, based on available building blocks, they were considered to be the easiest chemically accessible longer fragments, the octa- B(E)CDA'B'(E)'C' (20) and deca- B(E)CDA'B'(E)'C'DA'B'(E)'C' (21) were synthesized to study the length-dependent oligosaccharide-Ab recognition. \* indicates the residue linked at the nonreducing end (left end), whereas ' indicates the residue linked at the reducing end (right end) of the AB(E)CD biological RU. Di- and trisaccharides devoided of the E residue were either previously described or synthesized according to known procedures (22–26).

### Inhibition ELISA

Characterization of the saccharidic determinant recognized by the available mIgG was performed by measuring the mIgG-oligosaccharide interaction as follows. First, a standard curve was established for each mIgG tested. Different concentrations of the mAb were incubated overnight at 4°C on microtiter plates coated with purified *S. flexneri* 2a LPS at a concentration of 5  $\mu$ g/ml in carbonate buffer at pH 9.6 and subsequently incubated with PBS/BSA 1% for 30 min at 4°C. After washing with PBS-Tween 20 (0.05%), alkaline phosphatase-conjugated anti-mouse IgG was added at a dilution of 1/5,000 (Sigma-Aldrich) for 1 h at 37°C. After washing with PBS-Tween 20 (0.05%), the substrate was added (12 mg of *p*-nitrophenylphosphate in 1.2 ml of 1 M Tris-HCl buffer (pH 8.8) and 10.8 ml of 5 M NaCl). Once the color developed, the plate was read at 405 nm (Dynatech MR 4000 microplate reader). A standard curve OD =  $f(\text{Ab concentration})$  was fitted to the quadratic equation  $Y = aX^2 + bX + c$ , where  $Y$  is the OD and  $X$  is the Ab concentration. Correlation factor ( $r^2$ ) of 0.99 was routinely obtained.

Then the amount of oligosaccharides giving 50% inhibition of mIgG binding to LPS ( $\text{IC}_{50}$ ) was determined as follows. Each mIgG at a given concentration (chosen as the minimal concentration of Ab which gives the maximal OD on the standard curve) was incubated overnight at 4°C with various concentrations of each of the oligosaccharides to be tested in 1% PBS-BSA. Measurement of unbound mIgG was performed as described above using microtiter plates coated with purified LPS from *S. flexneri* 2a and the Ab concentration was deduced from the standard curve.

The recognition capacity of anti-LPS mIgG for LPS was determined as described above using various concentrations of LPS that were incubated in solution overnight at 4°C with the predefined concentration of each mIgG.  $\text{IC}_{50}$  was defined as the concentration of oligosaccharides required to inhibit 50% of mIgG binding to LPS.

### Semisynthetic glycoconjugates

Maleimide-activated: TT (batch FA 045644) was a gift from Sanofi-Pasteur (Marcy l'Etoile, France). It was stored at 4°C as a 39.4 mg  $\cdot$  ml $^{-1}$  stock solution in 0.05 N NaCl buffer. In a typical experiment, stock solution of TT (12 mg, 304  $\mu$ l, 0.08  $\mu$ mol) was diluted in 0.1 M PBS, pH 7.3 (296  $\mu$ l). To this solution was added ( $\gamma$ -maleimidobutyryloxy) sulfosuccinimide ester (Pierce) ( $3 \times 1.53$  mg,  $3 \times 50$  equivalent, dissolved in 60  $\mu$ l of  $\text{CH}_3\text{CN}/0.1$  M PBS, pH 7.3, 1:1) in three portions every 40 min. The pH of the reaction mixture was controlled (indicator paper) and maintained at 7–7.5 by addition of 0.5 M aqueous NaOH. Following an additional reaction period of 40 min, the crude reaction mixture was dialyzed against  $3 \times 2$  L of 0.1 M potassium phosphate buffer (pH 6.0) at 4°C using Slide-A-Lyzer dialysis cassettes (Pierce) displaying a membrane cutoff of 10 kDa. General procedure for the conjugation step: following dialysis, maleimide-activated TT in 0.1 M potassium phosphate buffer solution was reacted with each of the known synthetic *S*-acetylthioacetylated tri-, tetra-, penta-, deca-, and penta-decasaccharides (28) related to *S. flexneri* 2a O-SP in a 1:12 molar ratio, respectively. Reaction mixtures were buffered at a 0.5 M concentration by addition of 1 M potassium phosphate buffer (pH 6.0). Then  $\text{NH}_4\text{OH}/\text{HCl}$  (7.5  $\mu$ l of a 2 M solution in 1 M potassium phosphate buffer, pH 6) was added to the different mixtures and the couplings were conducted for 2 h at room temperature. The conjugated products were purified and stored as described previously (29). Hexose concentrations were measured by a colorimetric method based on the anthrone reaction using the corresponding oligosaccharides as standards (30). Protein concentrations were measured with the Lowry method using BSA as a standard (31).

### Biotinylated oligosaccharides

Conjugation of the known synthetic *S*-acetylthioacetylated tri-, tetra-, penta-, deca-, and penta-decasaccharides (28) related to *S. flexneri* 2a O-SP to EZ-link PEO-maleimide-activated biotin (Pierce) was run in phosphate buffer at pH 6.0 in the presence of hydroxylamine (32) and monitored by reversed-phase HPLC. Reversed-phase HPLC purification gave the target conjugates as single products, whose identity was assessed based on mass spectrometry analysis.

### Immunogenicity studies in mice

Seven-week-old BALB/c mice were immunized three times at 3-wk intervals, followed by a fourth injection 1 mo after the third one, with the equivalent of 10  $\mu$ g of oligosaccharide per mouse and per injection, in the absence of adjuvant, with the following glycoconjugates: B(E)C-TT, B(E)CD-TT, AB(E)CD-TT, [AB(E)CD] $_2$ -TT, or [AB(E)CD] $_3$ -TT. Control mice received TT alone using a dose equivalent to the maximum administered to mice receiving the glycoconjugates, i.e., 140  $\mu$ g/mouse and per injection. For the glycoconjugates incorporating the tri- or the tetrasaccharide and for the control mice, two independent experiments were performed using seven mice per group. For the three remaining glycoconjugates, three independent experiments were performed, two including 7 mice per group and one including 14 mice per group.

The Ab responses induced upon immunization were assessed 1 wk after the third and the fourth injections by ELISA. Purified LPS serotype 2a (14), biotinylated oligosaccharides corresponding to those incorporated into the glycoconjugates (this study), and TT were used as coated Ags to define the anti-LPS 2a, anti-oligosaccharide, and anti-TT Ab titer, respectively. Biotinylated oligosaccharides (0.5  $\mu$ g/well) were coated on plates previously incubated for 1 h at 37°C with avidin (1  $\mu$ g/well; Sigma-Aldrich). The amount of TT used for coating was 0.1  $\mu$ g/well. Anti-mouse IgG alkaline phosphatase-labeled conjugate (Sigma-Aldrich) was used as secondary Ab at a dilution of 1/5,000.

### Statistical analysis

Significant differences were established using Student's test. Values of  $p < 0.05$  were considered to be significant.

## Results

### Protective capacity of mIgG of different subclasses, specific for *S. flexneri* serotype 2a O-Ag

Different subclasses of IgG specific for LPS O-Ag are induced following natural infection with *Shigella* (33). To test whether all of the subclasses exhibit similar protective capacity, murine mIgG specific for serotype 2a determinants on the O-Ag and representative of each of the four murine IgG subclasses were obtained. Upon screening of hybridomas for their reactivity with LPS purified from *S. flexneri* serotype X, Y, 5a, 5b, 2a, 2b, 1a, 3a, respectively, five

mIgG exclusively recognizing the *S. flexneri* 2a LPS were selected: F22-4 (IgG1), D15-7 (IgG1), A2-1 (IgG2a), E4-1 (IgG2b), and C1-7 (IgG3). Each LPS-mIgG interaction was characterized by measuring the IC<sub>50</sub> that was shown to range from 2 to 20 ng/ml.

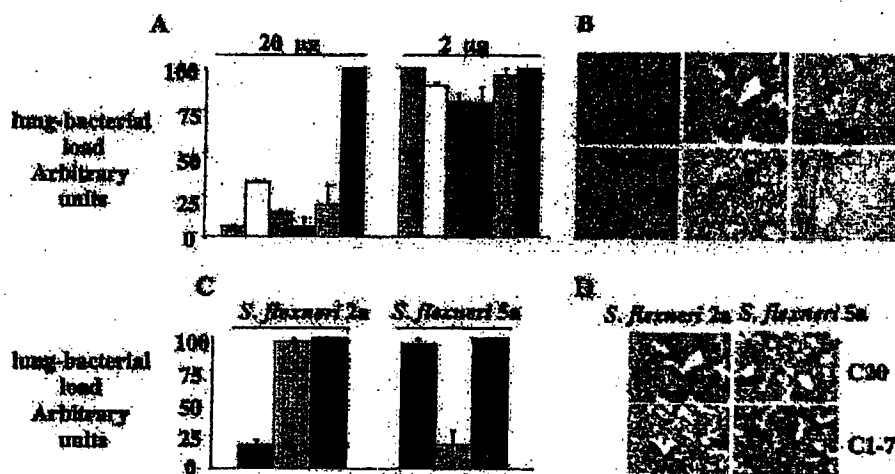
The protective capacity of the selected mIgG was analyzed using the murine model of pulmonary infection as previously described (10). In the absence of an intestinal model of experimental shigellosis in adult mice, this model has been shown to mimic the acute intestinal inflammation observed in humans. Indeed, mice infected i.n. with live virulent *Shigella* develop an acute bronchopneumonia characterized by a massive intra- and peribronchial polymorphonuclear (PMN) infiltrate, in addition to alveolitis (9, 10). Moreover, we recently demonstrated that this model is also relevant for mimicking the *Shigella*-induced immunomodulation of the host response observed during natural infection (Refs. 34 and 35; J. Gamelas-Magalhaes and A. Phalipon, unpublished observations). In addition, our previous data emphasize the relevance of this experimental model for testing the protective ability of mAbs that are expected to be effective in the intestinal environment. Indeed, the protective capacity of murine mAbs specific for *S. flexneri* serotype 5a primarily demonstrated in the mouse pulmonary model (10) has been confirmed using both in vitro and in vivo models of intestinal infection (Ref. 36; A. Phalipon and B. Corthésy, unpublished data).

Naive mice were administered i.n. with each of the purified mIgG before i.n. challenge with a sublethal dose of *S. flexneri*. Upon challenge, lung bacterial load in mice passively administered with 20  $\mu$ g of each of the mIgG specific for *S. flexneri* 2a LPS was significantly reduced in comparison to control mice receiving PBS (Fig. 2A). Upon passive transfer using 2  $\mu$ g of mIgG, only mIgG D15-7, A2-1, and E4-1 were shown to significantly reduce the lung bacterial load in comparison to control mice, but with much less efficiency than that observed using 20  $\mu$ g (Fig. 2A). As shown in Fig. 2B, reduction of lung bacterial load in mice receiving 20  $\mu$ g of mIgG was accompanied by a reduction of inflammation and therefore of subsequent tissue destruction. In comparison to con-

trol mice showing an acute bronchoalveolitis with diffuse and intense PMN cell infiltration (Fig. 2B, a and b) associated with tissue dissemination of bacteria throughout tissues (Fig. 2Bc), only restricted areas of inflammation were observed in Ab-treated mice, essentially in the intra- and peribronchial areas (Fig. 2B, d and e), where bacteria localized (Fig. 2B f). Following passive administration with 2  $\mu$ g of mIgG, inflammation resembled that of the control mice with a similar pattern of PMN infiltration and tissue destruction, in accordance with the very low, if any, reduction in lung bacterial load (data not shown). Moreover, the protection observed was shown to be serotype specific, as anticipated. Mice passively administered 20  $\mu$ g of mIgG C1-7 specific for *S. flexneri* 2a were protected against a homologous challenge, but not upon heterologous challenge with *S. flexneri* 5a bacteria (Fig. 2C). Similarly, mice receiving 20  $\mu$ g of mIgG C20, a *S. flexneri* serotype 5a-specific mAb of the same isotype as mIgG C1-7 (i.e., IgG3), showed significant reduction of lung bacterial load upon i.n. challenge with *S. flexneri* 5a, but not with *S. flexneri* 2a (Fig. 2C). In mice protected against homologous challenge, inflammation was dramatically reduced with a slight residual intra- and peribronchial PMN infiltrate (Fig. 2D, b and c). In contrast, in mice not protected upon heterologous challenge (Fig. 2D, a and d), inflammation and tissue destruction were similar to those observed in control mice (Fig. 2B, a and b).

#### Recognition of synthetic oligosaccharides by protective *S. flexneri* serotype 2a-specific mIgGs

A concentration of 1 mM was arbitrarily defined as the maximum ligand concentration to be used in ELISA inhibition assays. The binding of the five protective mIgGs to 23 synthetic mono- and oligosaccharides was evaluated in inhibition ELISA as described in *Materials and Methods*. None of the mono (A, B, and C are the same; D; E)- or disaccharides (AB; BC; CD; D'A; EC) showed any binding even when used at a concentration of 1 mM. Evaluation of trisaccharide recognition by testing D'AB, BCD, CDA', ABC, ECD, and B(E)C emphasized the unique behavior of mIgG F22-4, which was the only Ab showing measurable affinity for



**FIGURE 2.** Homologous (A and B) and heterologous (C and D) protection conferred by the different subclasses of mIgG specific for *S. flexneri* 2a serotype determinants. A, Mice receiving i.n. 20 or 2  $\mu$ g of purified mIgG, respectively, 1 h before challenge with a sublethal dose of virulent *S. flexneri* 2a bacteria. B, Histopathological study of mouse lungs. Upper row, Control mice; lower row, mice receiving mIgG. H&E staining: a and d, original magnification,  $\times 40$ ; b and e, original magnification,  $\times 100$ . Immunostaining using an anti-LPS Ab specific for *S. flexneri* serotype 2a: c and f, original magnification,  $\times 100$ . C, Mice receiving i.n. 20  $\mu$ g of each of the purified mIgG, C20, and C1-7 1 h before i.n. challenge with a sublethal dose of *S. flexneri* serotype 2a or serotype 5a bacteria. D, Histopathological study of mouse lungs. a and b, Mice receiving mIgG C20 specific for *S. flexneri* 5a and challenged with *S. flexneri* serotype 2a and 5a, respectively. c and d, Mice receiving mIgG C1-7 specific for *S. flexneri* 2a before challenge with *S. flexneri* serotype 2a and 5a, respectively. H&E staining, original magnification,  $\times 100$ . Lung bacterial load was expressed using arbitrary units with 100 corresponding to the bacterial count in lungs of control mice. SDs are represented ( $n = 10$  mice/group, three independent experiments).

Table I. Recognition of the synthetic oligosaccharides by the protective mIgGs\*

	F22-4	D15-7	A2-1	E4-1	C1-7
ECD <sup>a</sup>	179 ± 93	>1000	>1000	>1000	>1000
ECDA'	181 ± 102	>1000	>1000	>1000	>1000
ECDA'B'	354 ± 40	>1000	>1000	>1000	>1000
B(E)CD	5 ± 0.9	198 ± 79	>1000	87 ± 17	>1000
B(E)CDA'	2.5 ± 0.4	240 ± 65	340 ± 80	75 ± 9	400 ± 65
AB(E)C	>1000	>1000	>1000	>1000	>1000
D'AB(E)C	>1000	>1000	>1000	>1000	>1000
AB(E)CD	21 ± 9	490 ± 100	378 ± 24	287 ± 66	734 ± 200
B(E)CDA'B'(E')C'	0.22 +/- 0.02	60.8 ± 23	15 ± 5	12 ± 4.2	242 ± 124
D'AB(E)CDA'B'(E')C'	5 ± 1.4	11.9 ± 3.6	3 ± 1.8	4.4 ± 1.7	19 ± 4.5

\* Measurement of IC<sub>50</sub> (μM) by inhibition ELISA as described in *Materials and Methods*. SD is indicated. >1000 μM, indicates that no inhibition of LPS binding in the presence of the oligosaccharide tested at this concentration was observed.

<sup>a</sup> A = B = C: L-Rhamnopyranosyl residue; D: N-acetyl-D-glucosaminyl residue; E: D-glucopyranosyl residue. ' , The residue linked at the nonreducing end (left end) of the AB(E)CD RU; ' , the residue linked at the reducing end (right end) of the AB(E)CD biological RU.

such short oligosaccharides (Table I). ECD was the only trisaccharide recognized by F22-4, pointing out the crucial contribution of both the branched glucosyl residue (E) and the neighboring N-acetyl-glucosaminyl residue (D) to Ab recognition. This was supported by the absence of IgG recognition for AB(E)C or D'A-B(E)C at any of the concentrations used. Comparison of the recognition of the branched tetrasaccharide B(E)CD to that of the linear ECD indicated that rhamnose B, accounting for a reduction of the IC<sub>50</sub> by a factor of ~40, was also a key element in the recognition by F22-4, although of less impact than residue D, since B(E)C is not recognized. Indeed, B(E)CD was recognized by all of the protective mIgG, except A2-1 and C1-7, for which the minimal sequences necessary for recognition at a concentration below 1 mM were pentasaccharides AB(E)CD or B(E)CDA'. Extension of B(E)CD at the reducing end, yielding the branched pentasaccharide B(E)CDA', did not result in any major improvement of Ab binding for the other mIgGs. The minor, if any, contribution of reducing A to binding was also apparent when comparing F22-4 recognition of ECD and ECDA'. Further elongation at the reducing end, yielding ECDA'B' did not improve binding to F22-4.

Introduction of residue A at the nonreducing end of B(E)CD, leading to AB(E)CD, had a somewhat variable impact on Ab recognition with a positive effect in the case of A2-1 and C1-7 and a negative one by a factor ~2 to ~5 when considering the other Abs. Thus, for the recognition of short oligosaccharides, two families of mIgGs were identified: the first one, represented by F22-4, recognizing the ECD trisaccharide, and the second one, comprising the remaining four mIgGs, that recognized the same common

ECD sequence flanked by the B residue at the nonreducing end, elongated or not with residue A at either end. This observation was confirmed when measuring the recognition of extended oligosaccharides (Table I). Indeed, the decasaccharide D'AB(E)CDA'B'(E')C' showed the highest affinity for all Abs except F22-4. In the latter case, the octasaccharide B(E)CDA'B'(E')C' was the best recognized sequence with an IC<sub>50</sub> of 0.22 μM. Elongation to the decasaccharide by addition of D'A resulted in a loss of F22-4 recognition by a factor of ~20. Interestingly, recognition of the octa- and decasaccharides by the other mIgGs differed from that of F22-4. D15-7, and E4-1 behaved similarly. Extension of B(E)CDA' by B'(E')C' leading to the octasaccharide, and then by D'A leading to the decasaccharide, both resulted in improving Ab binding by a factor of ~4. Contribution of B'(E')C' to C1-7 binding appeared to be minor, whereas introduction of D'A resulted in an overall gain in binding of ~20. In the case of A2-1, addition of B'(E')C' to B(E)CDA' resulted in a gain in recognition by a factor of ~25, and subsequent elongation by D'A at the nonreducing end improved binding further by a factor of ~4. Thus, in general, elongating the oligosaccharide sequence increases the Ab affinity.

#### Molecular characterization of the protective *S. flexneri* serotype 2a-specific mIgG

To analyze whether the differences observed in the recognition of oligosaccharides by the mIgGs reflect differences in the structure of these mAbs, their CDRs were sequenced (Table II) (37). Only two V<sub>H</sub> and V<sub>L</sub> gene families were expressed among the five mIgGs studied. V<sub>H</sub> J606 (38) and VK24/25 (39) encoded F22-4

Table II. Comparison of the CDR sequences of mIgG specific for *S. flexneri* 2a

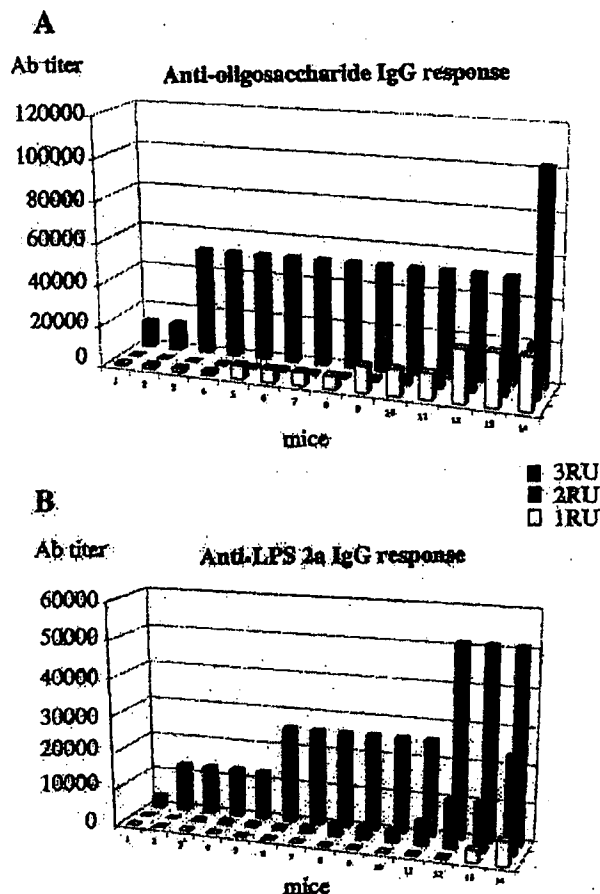
	H1	H2	H3
V <sub>H</sub>	31 35	52abc	100
A2-1	DYSLH	WINTETGEPA YADDFKG	YRYDG AY
C1-7	DYSIH	WINTKTGEPT YADDFKG	YDYAG FY
D15-7	YSSIH	WINTATGEPT YPDDFKG	YDYAG FY
E4-1	DYSMH	WVNTQTGEPS YADDFKG	YRYDG AH
F22-4	NYWMS	EIRLKSNDYATYYAESVKG	PM DY
V <sub>L</sub>	L1	L2	L3
	27abcde	50 56	89 97
A2-1	RATS SVGYIN	ATSNLAA	QQWSSDPFT
C1-7	SASS SVGYIH	DTSKLAS	QQWSSNPLT
D15-7	SASS SVGYIH	DTSKLAS	QQWSRNPLT
E4-1	RARS SVGYMN	ATSNQAS	QQWSSDPFT
F22-4	RSSKSLHSDGITYLY	HLSNLAS	AHNVELPRT

Kabat numbering of the CDRs (31). For the H chain, residues 31-35, 50-65, and 95-102 were defined as the first, second, and third CDR regions, respectively. For the L chain, residues 24-34, 50-56, and 89-97 were defined as the first, second, and third CDR regions, respectively.

$V_H$  and  $V_L$ , respectively. A2-1, C1-7, D15-7, and E4-1  $V_H$  genes were members of the VGAM3-8 family (40) and their  $V_L$  genes belonged to the VK4/5 family (39). The joining segment of the F22-4 H chain was encoded by JH4 (41), while A2-1, C1-7, D15-7, and E4-1 H chains shared the same diversity and joining segments, DSP2 (42) and JH3 (41), respectively. The joining segment for the L chain is encoded by JK1 (43) for F22-4, JK4 for A2-1 and E4-1, and JK5 for C1-7 and D15-7. For all mIgGs, the CDRs L2, L3, and H1 were similar to canonical classes (44), 1/7A, 1/9A, and 1/10A, respectively (45). For F22-4, the canonical forms of the loops L1 and H2 were similar to classes 4/16A and 4/12A, while those of the four other Abs belonged to classes 1/10A for L1 and 2/10A for H2. The CDR-H3 of A2-1, C1-7, D15-7, and E4-1 contained seven residues, including several aromatic ones, while the CDR-H3 of F22-4 was very short, made of only four amino acids with a proline residue in the first position. These results suggest that the unique behavior of F22-4 in recognizing the trisaccharide ECD, in comparison to the other mIgGs, could be related to a particular molecular structure.

#### Immunogenicity of semisynthetic glycoconjugates incorporating selected oligosaccharides

Among the different oligosaccharides tested, we selected 1) ECD since it was the shortest sequence recognized with an  $IC_{50}$  below 1 mM, at least by one of the five mIgGs (Table I); 2) B(E)CD since it was the tetrasaccharide recognized by three of five mIgGs in contrast to ECDA' recognized by F22-4 only and AB(E)C not recognized at all (Table I); and 3) AB(E)CD since it represents the biological O-Ag RU and was almost as well recognized by the five mIgGs as B(E)CDA (Table I). Besides, its synthesis was believed to be less demanding than that of B(E)CDA. Because longer sequences were shown to be better recognized than shorter ones, we tested the deca-saccharide  $[AB(E)CD]_2$  representing two biological RU and the penta-deca-saccharide  $[AB(E)CD]_3$  representing three biological RU, although they may not be the easiest targets when considering synthetic strategies. Our choice derived from the following observations: 1) the octa- and deca-saccharides used for the antigenicity study were chosen arbitrarily because they were readily available; 2) studies on short fragments have demonstrated the crucial input of reducing D in Ab recognition; and 3) the presence of nonreducing A was thought to be critical since terminal nonreducing residues of carbohydrate haptens may be immunodominant (46). The corresponding TT glycoconjugates were constructed (see *Materials and Methods*) and the average value for carbohydrate:protein ratio was shown to be 12. Intraperitoneal immunization of mice was performed using an equivalent of 10  $\mu$ g of oligosaccharide per dose without any adjuvant. The immunogenicity of the different glycoconjugates was assessed 7 days after the third and fourth immunizations, and the last boost was shown to significantly increase the anti-oligosaccharide and anti-LPS 2a IgG Ab titers (data not shown). ECD-TT neither elicited an anti-oligosaccharide IgG response nor an anti-LPS 2a IgG response. Anti-oligosaccharide Abs were induced by B(E)CD-TT, but no anti-LPS 2a IgG response was measured (data not shown). In contrast, the glycoconjugates incorporating 1, 2, or 3 RU raised both anti-oligosaccharide (Fig. 3A) and anti-LPS 2a IgG responses (Fig. 3B). However, we observed that the anti-LPS 2a IgG titer elicited as well as the number of mice responding was highly dependent on the hapten length (Fig. 3B). Whereas only 28.5% of mice responded to AB(E)CD-TT, 85% responded to  $[AB(E)CD]_2$ -TT and 100% to  $[AB(E)CD]_3$ -TT. In addition, mice immunized with the pentasaccharide elicited an anti-LPS 2a IgG titer significantly different from that induced by the pentadecasaccharide but not from that induced by the deca-saccharide ( $p = 0.005$  and 0.2, respec-



**FIGURE 3.** Immunogenicity of the selected oligosaccharides used as TT glycoconjugates. BALB/c mice were immunized three times at 3-wk intervals, followed by a last boost 1 mo later, with TT glycoconjugates incorporating 1, 2, or 3 RU using an equivalent of a 10  $\mu$ g/dose oligosaccharide, in the absence of adjuvant. Anti-oligosaccharide and anti-LPS 2a IgG responses were measured at day 7 after the last immunization by ELISA. The Ab titer was defined as the last serum dilution giving an OD of at least twice that obtained with sera of naive mice. Individual Ab responses are presented for one experiment including 14 mice and representative of three independent experiments. For both the anti-LPS and anti-oligosaccharide Ab responses, the difference between the groups of mice is statistically significant ( $p < 0.05$ ).

tively). Similarly, mice immunized with the deca-saccharide elicited an anti-LPS 2a IgG titer significantly lower than that elicited with the pentadecasaccharide ( $p = 0.0002$ ). No cross-reactivity against serotype 2a LPS and each of the selected oligosaccharides was detected in sera of control mice immunized with TT alone (data not shown). Taken together, these results demonstrate that the pentadecasaccharide  $[AB(E)CD]_3$  is as an accurate functional mimic of the O-Ag and, therefore, is a good candidate for the development of a chemically defined glycoconjugate vaccine against *S. flexneri* 2a infection.

#### Discussion

In view of developing a chemically defined glycoconjugate vaccine to *S. flexneri* serotype 2a, we have characterized the key elements of the serotype-specific immunodominant determinants carried by the O-Ag and analyzed their potential as functional mimics of the native Ag. Indeed, it is expected that more potent

anti-LPS-Abs will be induced using neoglycoproteins incorporating synthetic oligosaccharides accurately mimicking the O-Ag determinants targeted by protective Abs.

Five mIgGs specific for the serotype 2a of *S. flexneri* were characterized for their protective capacity, the amino acid sequences of their CDRs, and the oligosaccharide determinants they recognize on the O-Ag. It is noteworthy that a large number of O-Ag-specific anti-*Shigella* mAbs were produced for diagnosis purposes (47-57). However, only a few of them were precisely characterized in terms of protective capacity, recognition pattern, or molecular structure (48, 58-60). To obtain mIgG, hybridoma cells were selected on the basis of their secretion of mAb recognizing determinants specific for the *S. flexneri* serotype used for immunization, i.e., serotype 2a or 5a, respectively. During screening, most of the hybridoma cells tested (~90%) were shown to secrete serotype-specific mAbs. This result differs slightly from previous reports on the isolation of mAbs directed to determinants common to several *S. flexneri* serotypes including 2a and 5a (53, 54). It may be explained by recent new insights on *S. flexneri* O-Ag conformation in the case of *S. flexneri* serotype 5a demonstrating the impact of the E residue specifying the serotype on the overall conformation of the O-Ag (61). Analogously, we may reasonably hypothesize that this residue also protrudes exquisitely from the surface of the O-Ag in the case of serotype 2a, and being repeatedly exposed at the bacterial surface, preferentially triggers B cell receptor-mediated recognition, thus leading to the induction of a predominant anti-serotype specific Ab response. In favor of this hypothesis is the demonstration that during natural infection in endemic areas where several *Shigella* species and serotypes coexist, the mucosal Ab response is predominantly directed against serotype-specific determinants (2, 62).

Importance of the E residue and its surrounding sugars is again emphasized in the current study. Indeed, by measuring oligosaccharide-mAb interaction in inhibition ELISA, we showed that the *S. flexneri* 2a O-Ag exhibits an intrachain immunodominant determinant comprising the trisaccharide ECD as the minimal sequence required for inhibiting mIgG-LPS recognition. Depending on the mAb, additional flanking residues at the nonreducing or reducing end of ECD are required for optimal recognition at ligand concentrations below 1 mM. Since EC is not recognized by any of the protective mAbs, binding of F22-4 to ECD points out to the key contribution of the *N*-acetyl-glucosamine D residue in the interaction. A F22-4 closely related binding pattern has been reported for mAb SA-3 specific for the cell wall polysaccharide of group A *Streptococcus* whose RU is a branched trisaccharide (63). Indeed, Abs to branched polysaccharides often recognize the branching point as shown for anti-*Salmonella* serogroup B mAb Sc115-4 (64) and for mIgG C20 specific for *S. flexneri* 5a O-Ag. The latter, shown in the present article to be protective against a homologous but not a heterologous strain (Fig. 3), recognizes the branched A(E)B trisaccharide epitope (A. Phalipon, unpublished data).

In accordance with the unique recognition pattern of F22-4, we showed that the CDR sequences of this mAb completely differ from those of the four remaining ones. It is noteworthy that the F22-4 CDRs H1, H2, L1, and L2 are quite similar in sequence and/or length to those of SYA/J6, an IgG3 specific for *S. flexneri* serotype Y, one of the few antibacterial polysaccharide Ab for which x-ray studies of Fab-oligosaccharide interactions have been determined (65). In contrast, the H3 loops, which are the major key of Ab diversity, differ strongly. The CDR-H3 comprises nine and four amino acids for SYA/J6 and F22-4, respectively. SYA/J6 is an example of a groove-like site for binding of an internal ABCDA' oligosaccharide epitope repeatedly exposed on the helical *S. flexneri* Y O-Ag (60) and its base which includes three Gly

residues shows the torso-bulged structure (66). In the case of F22-4, the H3 loop, which can only form a short hairpin, would probably allow a more open binding site that could accommodate the branched E residue.

For the other mIgGs, B(E)CD is the minimal sequence required for recognition. These mAbs bind intrachain epitopes, as indicated by the fact that D15-1 and E4-1 bind to B(E)CD slightly better than to AB(E)CD. This is strongly supported by the increased recognition, by all mIgGs, of the octasaccharide and decasaccharide having a B and a D residue at their reducing end, respectively, in comparison to that of AB(E)CD. In this regard, the carbohydrate-binding specificity of this family of Abs resembles that of a panel of Abs specific for the well-studied Gram-positive group A *Streptococcus* cell wall polysaccharide, that has a branched trisaccharide RU (67). It is somewhat puzzling to note that although their recognition of the shorter oligosaccharides slightly differs, all of the mIgGs fall into the same pattern of binding when considering the decasaccharide. In accordance with their similarities in oligosaccharide recognition, D15-1, E4-1, A2-1, and C1-7 exhibit similar CDR sequences for both their H and L chains. Further structural analysis of these mIgGs in complex with oligosaccharides will determine which mAb residues are directly involved in Ag binding and will identify the contribution of each sugar to Ab recognition.

As previously reported using the same experimental model for a murine mAb of the A isotype (mIgA) specific for *S. flexneri* serotype 5a mimicking the secretory IgA-mediated mucosal response (10), we demonstrate here the protective capacity of serotype-specific mIgGs, regardless of their subclasses, in controlling homologous infection with *S. flexneri* 2a. A similar result is reported for mIgG C20 specific for *S. flexneri* serotype 5a. In both cases, no cross-protection was shown to occur. With the limits of the experimental model, these data contribute to a better understanding of the role of systemic vs mucosal Ab responses in protection against reinfection by emphasizing the contribution of serotype-specific systemic IgG response in protecting the host against *Shigella* reinfection as earlier hypothesized (68). This is in accordance with previous results indirectly demonstrating the protective role of the systemic response upon vaccination on the field using a detoxified *S. sonnei* LPS-based conjugate administered parenterally and eliciting mainly a specific anti-LPS IgG response (4).

By testing the immunogenicity of selected oligosaccharides based on our antigenicity data, we demonstrated that not all of the selected antigenic sequences accurately mimic the natural Ag. In particular, the tetrasaccharide B(E)CD was identified as an immunodominant epitope on the O-Ag, and immunization with the corresponding TT conjugate induced a high titer of anti-B(E)CD Abs, indicating that this oligosaccharide was immunogenic in mice. However, in contrast to other bacterial systems for which protein conjugates incorporating fragments shorter than 1 RU of the surface capsular polysaccharide (CP) were shown to induce anti-CP Abs (69, 70), B(E)CD-TT failed to induce any detectable anti-LPS Abs, demonstrating that this short sequence was not a functional mimic of the O-Ag. An analogous situation was observed earlier for *S. pneumoniae* type 6B semisynthetic glycoconjugates evaluated in mice, although conjugates incorporating haptens smaller than 1 RU induced anti-CP protective Abs in rabbits (71). Interestingly, AB(E)CD-TT, incorporating the exact biological RU, was able to induce anti-LPS Abs in mice although with a poor efficacy. These results support those obtained with a series of *Shigella dysenteriae* type 1 glycoconjugates bearing haptens differing by length and reporting that a minimum of 2 RU were needed to induce high titers of anti-LPS Abs (5). In contrast, our data differ from those published on *S. pneumoniae* type 14 (72) and type 3 (6)

and emphasize that bacterial PS should be dealt with on a case-by-case basis. The most striking observation in our study was the strong enhancement of the anti-LPS Ab titer resulting from elongation of the hapten length, when going from one biological RU, to 2 and 3 RU. This is in accordance with previous reports emphasizing that better immunogenicity can be obtained with longer saccharidic haptens (5, 46) and former assumptions that a minimum of 2 RU was necessary for inducing a strong anti-polysaccharide Ab response.

As hypothesized earlier (68), and subsequently confirmed in human studies using classical glycoconjugates based on detoxified *S. sonnei* LPS (4), the presence of anti-LPS IgG appears as a reliable marker to predict protective immunity induced by parenterally administered glycoconjugate vaccines. According to this assumption, several groups have gone through clinical trials (73–75), and others are considering doing so (5). We are, therefore, confident in the efficacy of the pentadecasaccharide mimic we identified in inducing protective immunity. The parameters influencing the immunogenicity of the glycoconjugate are currently being studied to envision in the near future a Phase I clinical trial.

Since no data are available, so far, a question that remains open is the feasibility of the semisynthetic strategy for the development of multivalent glycoconjugate vaccines. Actually, the approach we have undertaken may appear more time-consuming than the combination of the relevant classical polysaccharide-protein conjugates, involving polysaccharides purified from the different prevalent strains, detoxified if required, and subsequently coupled to an appropriate carrier. However, we assume that in addition to potential advantages previously mentioned (cf. Introduction), the chemically defined approach is particularly suited for the construction of a multivalent *S. flexneri* vaccine. Indeed, *S. flexneri* serotypes only slightly differ in their O-Ag structure and future synthetic strategies could benefit from knowledge gained from our work on serotype 2a. In-house ongoing studies will contribute to the assessment of the accuracy of our assumption.

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## Disclosures

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Original article

## Vaccination against shigellosis: is it the path that is difficult or is it the difficult that is the path?

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### Abstract

Following several decades of research, there is not yet a convincing vaccine against shigellosis. It is still difficult, in spite of the breadth of strategies (i.e. live attenuated oral, killed oral, subunit parenteral) to select an optimal option. Two approaches are clearly emerging: (i) live attenuated deletion mutants based on rational selection of genes that are key in the pathogenic process, and (ii) conjugated detoxified polysaccharide parenteral vaccines, or more recently conjugated synthetic carbohydrates. Some of these approaches have already undergone phase I and II clinical trials with promising results, but important issues have also emerged, particularly the discrepancy between colonization and immunogenic potential of live attenuated vaccine candidates depending upon the population concerned (i.e. non endemic vs. endemic areas). Efforts are needed to definitely establish the proof of concept of these approaches, and thus the need for clinical trials which should also soon explore the possibility to associate different serotypes, in response to serotype specific protection against shigellosis. More basic research is also required to improve what we can still consider as first-generation vaccines, and to explore possible new paradigms including the search for cross-protective antigens.

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Shigellosis (bacillary dysentery) is endemic throughout the planet, although essentially a major health concern in its most impoverished areas, particularly in the pediatric population between 1 and 5 years old. It can be caused by any serotype belonging to four groups: group A (*Shigella dysenteriae*), group B (*Shigella flexneri*), group C (*Shigella boydii*), and group D (*Shigella sonnei*). The serotype 1 of *S. dysenteriae* (SD1) emerges as one of particular concern, due to its expression of the Shiga toxin, a potent cytotoxin that not only aggravates the intestinal symptomatology, but also causes

major systemic complications such as the Hemolytic Uremic Syndrome (HUS). *S. sonnei* incidence tends to increase over other groups as living standards improve, thus dominating as an endemic strain in western countries. The disease is characterized in its classical form, by a short period of watery diarrhea with increasing intestinal cramps and general malaise, followed by the appearance of a dysenteric syndrome that comprises intestinal cramps and tenesmus, leading to permanent emission of bloody, often mucopurulent stools. Acute complications may occur in absence of quick antibiotic treatment, such as toxic megacolon, peritonitis, and septicemia that is mostly observed in severely malnourished children. Conversely, repeated shigellosis episodes may lead to severe malnutrition, thus a vicious circle. When poor conditions are concentrated in a single epidemiological crisis, like in refugee camps, the attack and mortality rates may be quite high, as observed in Goma, Zaire, in 1994 in the course of an SD1

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epidemic. Projections based on methodologically convincing epidemiological studies from the three previous decades allowed, back in 1999, to evaluate the number of cases of shigellosis to 169 million per year, with a death rate ranging between 500,000 and 1.1 million, 69% being children below 5 years in the developing world [1]. These impressive figures have undoubtedly led the community to realize that shigellosis is a high-impact disease, particularly in the poorest populations. The WHO has set it at the top of its priority list, along with ETEC, for the development of a vaccine, and this has recently emerged as a “*Shigella*-ETEC vaccine initiative” by the Bill & Melinda Gates Foundation. Current figures may not be that high however, although the epidemiological situation is evolving and figures are lacking in key areas, particularly in Africa. Recent surveys indicate that in general, the incidence of diarrheal diseases remains stable worldwide, although mortality shows sustained decrease, being currently evaluated at a level of 4.9/1000 per year [2]. A recent epidemiological survey conducted in six Asian countries [3] has established that shigellosis was likely to be following a similar trend with a stable incidence of cases (4.6% of cases of diarrhea), and decreased severity and mortality. The rationale for this switch in disease profile is still unknown. Several modes of explanation may be envisioned, such as better nutrition and hygiene paralleling economic development of the Asian continent, absence of current epidemics of SD1, better education of mothers, improvement of primary health care, and extended use of ORS and antibiotics. The issue of antibiotics is likely to be an important one. Beyond the possibly positive impact of free, uncontrolled use of antibiotics on the disease profile at this stage, one may just as soon have to face a new crisis associated with multidrug resistance. In some areas, the prevalence of strains resistant to all first-line antibiotics, including fluoroquinolones, reaches 5% and is clearly on the rise. It is also unlikely that the epidemiological situation in Asia can be generalized, thus there is a need for an exhaustive evaluation of the incidence of shigellosis, particularly in the sub-Saharan part of the African continent. Current economic stagnation and frequent social instability are creating conditions for shigellosis to remain a leading cause of morbidity and mortality. In order to facilitate such studies, there is a need for efficient and durable surveillance networks benefiting from good microbiological expertise and novel quick, reliable, and robust diagnostic tools such as immunochromatographic dipsticks that could be used directly on fecal samples [4].

All elements being considered, including the permanent risk of massive re-emerging epidemics, the need for a *Shigella* vaccine clearly remains. Its major target would be the pediatric population of the developing world, essentially infants around the age of 1 year, and possibly also the elderly population that represents the other peak of disease susceptibility. Such a vaccine could also benefit travelers to high-risk areas, particularly those working or intervening in these areas, e.g. members of NGOs, army personnel, etc.

*Shigella* infection prevents disease during subsequent exposures. Lipopolysaccharide (LPS), the major bacterial surface antigen, is the main target of the host adaptive

immunity. Anti-LPS antibodies (Abs) are elicited upon infection, both locally as secretory IgA (SIgA), and systemically as serum IgG. Ab-mediated protection has been shown to be mostly serotype specific [5], pointing to the O-specific polysaccharide moiety of LPS, also termed O-antigen (O-Ag), as the target of the protective immune response. Indeed, *Shigella* serotypes are defined by the structure of their O-Ag repeating unit (RU) [6].

This has been a strong incentive to considering that protection was an achievable goal with an oral vaccine reproducing key steps of the natural infectious process. Still, natural protection is not absolute and rather short lasting, and again, essentially serotype specific [7]—not necessarily good news for *Shigella* vaccine developers. Nevertheless, phase III trials carried out in Yugoslavia in the 1960s [8], using Streptomycin-dependent (Smd) mutants of *S. flexneri* and *S. sonnei*, had shown that oral vaccination was an achievable goal. This has remained over the years the gold standard, even though reversion of the mutation, in some cases, had illustrated the need for an association of attenuated mutations consisting in gene deletion, whose selection would be rationally based on the increasing knowledge in the pathogenic mechanisms of *Shigella* [9]. Following these encouraging initial results, attempts were indeed made at rationally attenuating virulence of candidate strains representing the most frequently isolated serotypes, such as *S. flexneri* 2a and *S. sonnei*, as well as *S. dysenteriae* serotype 1, due to severity of cases. Two major strategies have been considered: (i) altering key metabolic pathways affecting bacterial growth in tissues, or (ii) knocking out virulence genes selected upon their expected capacity to affect one or several key steps of the infectious process. Some recent vaccine candidates have combined both approaches. An initial candidate belonging to the first category, an *aro* mutant of *S. flexneri* (SFL124) expressing the *S. flexneri* group antigen, was constructed in an attempt to obtain cross-protection. This mutant appeared too attenuated, thus very well tolerated by volunteers in clinical trials, but weakly immunogenic [10,11]. This vaccine candidate raised an important issue regarding the bases of its attenuation. It is likely that the wild-type strain that had been selected was already weakly pathogenic, therefore its further attenuation by *aro* mutation likely accounted for insufficient colonization potential and immunogenicity. A recent review has stressed the need to confirm full pathogenicity of the strains that serve as a basis for vaccine construction [7]. This is ethically complicated, but the mere isolation from a patient may not guarantee that the isolate shows “optimal” pathogenicity. Other metabolic mutations have been considered, particularly *guaAB* that introduces a severe auxotrophy impairing synthesis of nucleic acids [12], as well as mutations impairing the strain’s capacity to scavenge ferric iron ( $\text{Fe}^{3+}$ ), a property required to compete for vital  $\text{Fe}^{3+}$ , via the production of siderophores (i.e. aerobactin or enterochelin), against iron-chelating molecules of mucosal surfaces (i.e. lactoferrin), or of tissues (i.e. aerobactin) [13]. The most recent *Shigella* vaccine candidates have undergone a combination of metabolic and virulence mutations. This combination can lead to various

degrees of attenuation. Current vaccine candidates, on these bases, can fall into the category of weakly attenuated or strongly attenuated strains.

In the category of weakly attenuated candidates belong *icsA*-based mutants. *IcsA* is an outer membrane protein of *Shigella* that nucleates cellular actin, thereby allowing intracellular motility and cell to cell spread of the microorganism. Mutation in this gene impairs the capacity of *Shigella* to spread extensively in the epithelium, away from its initial site of entry [14]. It has been shown that such mutants were directly targeted to colonic solitary nodules, the actual inductive sites of the mucosal immune response [15]. Combined with a deletion of the aerobactin system (*iuc iut*), in *S. flexneri* 2a, *icsA* has provided a vaccine candidate (SC602, Institut Pasteur) that has undergone phase I and II clinical trials (Walter Reed Army Institute of Research and US Army Institute for Research in Infectious Diseases) whose results were considered encouraging in Western volunteers [16]. In brief, the strain was strongly immunogenic, eliciting a high percentage of circulating plasmacytes producing anti-LPS IgA by the ELISPOT assay, although showing residual reactogenicity with limited fever and diarrhea in about 15% of the recipient volunteers. Moreover, when vaccinees who had received a dose of  $10^6$  CFU as vaccine inoculum were challenged with a wild-type, pathogenic *S. flexneri* strain of similar serotype, they appeared fully protected against dysentery, and subsequent studies carried out in the USA and Israel demonstrated the absence of accidental transmission [17]. In short, this was a quite encouraging series of studies that certainly confirmed the concept that a live attenuated *Shigella* vaccine is an achievable goal. A  $\Delta$ *icsA* *S. sonnei* vaccine candidate constructed by scientists at WRAIR (WRSS1) showed similar results with regard to tolerance and immunogenicity [18]. More recently, a *S. dysenteriae* 1 vaccine candidate (SC599, Institut Pasteur) has been tested in phase I and II trials (Saint George Vaccine Institute, London, UK, and Centre de Vaccinologie Cochlin-Pasteur, Paris, France). In this strain, three deletions have been introduced:  $\Delta$ *icsA*,  $\Delta$ *ent fep fex* (genes encoding the enterochelin system), and  $\Delta$ *astA*, the gene encoding the catalytic subunit of Shiga toxin. Unlike its *S. flexneri* and *S. sonnei*  $\Delta$ *icsA* counterparts, this strain has shown good tolerance, limited systemic immunogenicity (as judged by seric IgM, IgG and IgA titers), and average to good mucosal immunogenicity as judged by percentage of anti-LPS IgA measured by ELISPOT, in comparison to SC602 and WRSS1 (in press, and in preparation). In the absence of clear correlates of protection, it is currently difficult to anticipate the potential of this family of vaccines for the future. This is a particularly important issue, as the serotype-dependent nature of protection would necessitate further construction of strains, particularly *S. flexneri* 3a, 1b and 6, in order to cover a broader spectrum of serotypes [19], and testing of a combination of these strains to address issues such as interference. Last but not least, only a phase III efficacy trial conducted in an endemic area may provide the final piece of information required to validate the approach. To add to the difficulty, SC602 was tested in a phase II trial in a highly endemic area of Bangladesh (collaboration between ICDDR,B,

AFRIMS, and WHO). The strain showed excellent tolerance in all age categories, including 1-year old infants with inocula up to  $10^7$  CFU (unpublished data). On the other hand, colonization appeared limited and immunogenicity very weak. Beyond possible technical issues, it remains that one dose of such  $\Delta$ *icsA*-based vaccine appears immunogenic and possibly protective in (*Shigella*-naïve) Western populations, but clearly shows less immunogenicity and weak capacity to colonize in endemic areas. Several possibly combined hypotheses may account for this issue: the protective role of breast feeding against the vaccine strain in infants, the high level of innate stimulation of the intestinal mucosa by recurrent enteric infections in a highly endemic zone, thereby severely affecting the capacity of the vaccine strain to colonize the mucosa (in this context, the nature of the selected mutation may need to be discussed), the high exposure of children, at an early age, to multiple enteric pathogens, including the most prevalent serotypes of *Shigella*, thus a quickly acquired status of adaptive immunity. In any event, these observations are important to consider because they are very unlikely to apply only to this particular category of vaccine candidate. Considering at least two oral doses as a possible solution to discuss, it would require a second phase II study in similar epidemiological conditions.

To the category of strongly attenuated strains belongs a series of strains constructed at the Center for Vaccine Development (University of Maryland). In the most recent generation of vaccine constructs, a *S. flexneri* 2a strain has undergone a *guaAB* mutation that has been combined with a *sen* and a *set* mutation, thereby knocking out the genes encoding two putative enterotoxins of this serotype. A strain named CVD 1208 has been tested in a phase I trial [20,21]. The tolerance appeared excellent, including the lack of residual diarrhea, validating the elimination of *Sen* and *Set* expression, thereby allowing administration of vaccine doses up to  $10^9$  CFU without side effects. At such doses, systemic and mucosal responses reached good levels, similar to those observed with SC602 with a  $10^4$  CFU inoculum, if one tries a comparison [16].

This is clearly an alternative option that also needs to be validated in further trials, including in the field.

In spite of the encouraging results observed in the course of these different studies, issues clearly remain such as:

- Will it be possible to immunize with a single oral dose?
- What is the acceptable limit of serotype numbers to be introduced in a multivalent oral vaccine, considering the increasing serotype diversity observed depending on the region considered?
- Should the current strategies of vaccine design take into account the most recent evidence that *Shigella* is able to strongly interfere with the innate and adaptive immune response of the host, thereby creating an immunosuppressive environment that is not questionable in a vaccine concept? ([9,22]; Gamelas Magalhães et al., submitted)

Subunit vaccines based on the use of the major protective antigen, i.e. LPS, and administered parenterally are an

alternative option for the development of a *Shigella* vaccine. However, LPS cannot be used as a parenterally administered immunogen, due to its highly toxic lipid A. Strategies involving constructs whereby LPS toxicity is masked, or LPS analogues devoided of toxicity, were investigated instead. Similarly to the successful conversion of bacterial capsular polysaccharides from T-cell independent antigens (Ag) to T-cell dependent ones [23–25], the non-toxic acid-detoxified *S. flexneri* 2a LPS (pmLPS) was turned into a potent T-cell dependent immunogen through its covalent coupling to a protein carrier. Since the early 1990s, several *S. flexneri* 2a pmLPS–protein conjugates have been shown to be safe and immunogenic in adults [26,27] as well as in young children [28]. The most encouraging results were obtained with a *S. sonnei* pmLPS–rEPA conjugate vaccine administered parenterally to young adults, showing protection in about 75% of the vaccinees during a *S. sonnei* outbreak [29]. Nevertheless, *Shigella* pmLPS–protein conjugate vaccines remain complex constructs obtained from randomly activated pmLPS. Potential loss of antigenicity may occur during detoxification and/or coupling to the carrier. Thus, accurate controls of these two crucial steps are required to ascertain both the complete removal of LPS toxicity and maintenance of LPS antigenicity. In addition, appropriate consideration should be given to the increasing requirements from regulatory agencies for always better-defined molecules to be used in humans.

An alternative to conventional pmLPS–protein conjugates is the use of synthetic mimics of the bacterial O-Ag. Indeed, following early reports in the 1940s [30], increasing evidence supports the concept that carbohydrate epitopes, which are made of short oligosaccharide (OS) sequences, are immunogenic in animal models once conjugated to appropriate carriers [31–33]. Most importantly, recent and anticipated future improvements in glycochemistry are expected to give access to better-defined and standardized complex carbohydrates. Thus, besides the search for peptide mimics [34–37], the use of OS mimicking the carbohydrate determinants recognized by anti-O-Ag protective monoclonal antibodies (mAbs) has been developed [38,39]. Such mimics are expected to induce a protective anti-LPS Ab response when appropriately presented to the immune system. Along this line, a new vaccine candidate targeting *S. flexneri* 2a infection has recently emerged [40]. This synthetic OS-based conjugate was designed in order to obey to the following rules: (i) the use of carbohydrate haptens suitable for single-point attachment onto a carrier to overcome the limitations due to LPS random chemical modifications and/or detoxification; (ii) the control of various parameters such as the length and nature of the carbohydrate hapten, its loading onto the carrier, as well as the choice of the carrier, to allow the design of glycoconjugates with optimal immunogenicity. It was issued from a four-step process encompassing (i) identification of the protective *S. flexneri* 2a epitopes, (ii) conception of the candidate glycoconjugates, (iii) study of the immunogenicity of the glycoconjugates in mice, and (iv) when appropriate, analysis of the protective efficacy of the anti-*S. flexneri* 2a LPS Abs induced by the glycoconjugates.

A rational strategy was undertaken for the identification of protective *S. flexneri* 2a epitopes. The methyl glycosides of di- to pentasaccharides representing frame-shifted fragments of the basic *S. flexneri* 2a O-Ag RU (a branched pentasaccharide AB(E)CD as shown in Fig. 1), together with an octa- and a decasaccharide, were synthesized by multi-step chemical synthesis [41–46]. Analysis of the contribution of each monosaccharide residue to the recognition of *S. flexneri* 2a LPS by several serotype 2a-specific protective mAbs showed that most mAbs bind internal epitopes repeatedly exposed on the LPS. In addition to outlining the key role played by ECD in Ab recognition, B(E)CD was shown to represent an immunodominant protective determinant. However, since chain elongation improved mAb binding, a deca- and a pentadecasaccharide representing 2 and 3 biological RU, respectively, were synthesized [47] in addition to selected short haptens [48].

Upon coupling of several OSs including ECD, B(E)CD, AB(E)CD, [AB(E)CD]<sub>2</sub>, and [AB(E)CD]<sub>3</sub>, to the classical carrier protein, tetanus toxoid (TT), by controlling both the coupling chemistry and the OS loading, immunogenicity of the resulting glycoconjugates was investigated in mice. [AB(E)CD]<sub>3</sub> clearly appeared as the best sequence among those tested for the induction of anti-LPS IgGs specific for *S. flexneri* 2a. Indeed, AB(E)CD and [AB(E)CD]<sub>2</sub> were eliciting a significant, albeit lower, anti-LPS IgG response [40]. More importantly, IgGs induced by the glycoconjugate incorporating [AB(E)CD]<sub>3</sub> were shown to be protective, and protection was clearly shown to be dependent on the anti-LPS Ab titer [49] (Phalipon et al., submitted).

A phase I clinical trial is envisioned and preclinical investigation is under way, the major goal being the production of GMP batches of the selected conjugate by companies expert in the synthesis of complex carbohydrates and/or polysaccharide–protein conjugates. The good news is that the multi-step chemical synthesis leading to the obtaining of [AB(E)CD]<sub>3</sub> has been recognized as feasible at large scale and reasonable cost, suggesting that the semi-synthetic glycoconjugate vaccine approach remains an attractive option that fulfills most of the WHO requirements concerning the availability of vaccines for developing countries.

Further development will integrate the need for multi-valency, and a lot of effort is currently invested in the identification of a cross-protective antigen. Besides the testing of new routes of systemic delivery, immunization is being investigated.

Recent evidence indicates that some structures involved in the pathogenesis of *Shigella*, the protein components of which are highly conserved throughout groups and serotypes, could be considered as antigens that may elicit neutralizing Abs expected to disarm *Shigella* in the course of its pathogenic process. Such antigens that may provide reasonable—if not

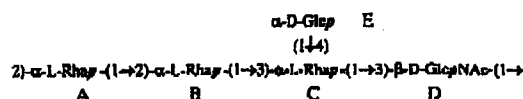


Fig. 1. Structure of the basic repeating unit of the O-antigen moiety of *S. flexneri* 2a LPS [6].

complete—cross-protection could also be used as carriers for the major polysaccharide-based serotypes, and thus a creative combination able to generate a new paradigm of protection against the diverse *Shigella* serotypes.

As a matter of fact, one of the dominant issues soon to appear will be the capacity to obtain the strongest possible immunogenicity combined with the highest level of cross-protection, in the simplest possible vaccine preparation.

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# A Synthetic Carbohydrate-Protein Conjugate Vaccine Candidate against *Shigella flexneri* 2a Infection<sup>1</sup>

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The protective Ag of *Shigella*, the Gram-negative enteroinvasive bacterium causing bacillary dysentery, or shigellosis, is its O-specific polysaccharide (O-SP) domain of the LPS, the major bacterial surface component. As an alternative to the development of detoxified LPS-based conjugate vaccines, recent effort was put into the investigation of neoglycoproteins encompassing synthetic oligosaccharides mimicking the protective Ags of the O-SP. We previously reported that when coupled to tetanus toxoid via single point attachment, a synthetic pentadecasaccharide representing three biological repeating units of the O-SP of *Shigella flexneri* 2a (SF2a), one of the most common *Shigella* serotypes, elicits a better serum anti-LPS 2a Ab response in mice than shorter synthetic O-SP sequences. In this study, we show that the pentadecasaccharide-induced anti-LPS 2a Abs protect passively administered naive mice from *Shigella* infection. Therefore, this three repeating units sequence, which is recognized by anti-SF2a sera from infected patients, acts as a functional mimic of the native polysaccharide Ag. Analyses of parameters influencing immunogenicity revealed that an investigational SF2a vaccine displaying a pentadecasaccharide:tetanus toxoid molar loading of 14:1 triggers a high and sustained anti-LPS Ab response, without inducing anti-linker Ab, when administered four times at a dose corresponding to 1  $\mu$ g of carbohydrate. In addition, the profile of the anti-LPS Ab response, dominated by IgG1 production (Th2-type response), mimics that observed in human upon natural SF2a infection. This synthetic carbohydrate-based conjugate may be a candidate for a SF2a vaccine. *The Journal of Immunology*, 2009, 182: 2241–2247.

Parenterally administered polysaccharide-protein conjugate vaccines have been shown to be more effective than the first generation of polysaccharide vaccines made of purified polysaccharide Ags. Interestingly, all licensed second generation polysaccharide vaccines target capsulated bacteria (1), and there is no commercialized LPS-based conjugate vaccine. The toxicity of lipid A (i.e., endotoxin), a major component of LPS, which precludes its use in conjugate vaccines, largely accounts for the current situation. LPS-detoxification is a prerequisite to the development of LPS-based conjugate vaccines. Despite this difficulty, vaccine candidates derived from LPS lacking full-length lipid A-chains are under investigation (2–6). Alternatively, progress in glycochemistry has opened the way to third generation polysaccharide vaccines, namely, synthetic carbohydrate-protein conjugate vaccines (7–10).

*Shigella*, a Gram-negative enteroinvasive bacterium causing shigellosis, is a major public-health concern worldwide (11–13).

Shigellosis is endemic in areas with a low level of hygiene, mainly targeting children under 5 years of age. There is as of yet no broadly licensed vaccine for this common serious disease (14). *S. flexneri* 2a (SF2a),<sup>4</sup> a member of *Shigella* Group B, remains the most common *Shigella* worldwide (12). In addition, in countries where the disease is endemic, several *S. flexneri* serotypes are isolated. However, their distribution varies based on geographical areas (12). Serological classification is based on the nature of the repeating unit (RU) (15, 16) of the O-specific polysaccharide (O-SP) moiety of LPS, which acts as a major virulence factor for *Shigella*, providing the bacterium with resistance to host defense mechanisms (15). Protection induced by natural infection is considered serotype-specific, pointing to the O-SP as the major target of protection. Early on, the protective role of anti-O-SP serum IgGs, through killing of *Shigella* inoculum on the epithelium surface of small intestine, was hypothesized (2, 17). It was subsequently supported by clinical data (18) and reports on the immunogenicity of *Shigella* detoxified LPS-protein conjugates in adults (19) and in young children (20). In addition, it was recently suggested that such Abs might be curative (4).

We initiated a program aimed at developing a third generation carbohydrate-based vaccine against SF2a infection. The strategy is based on the use of synthetic oligosaccharides (OSs), acting as efficient functional SF2a O-SP mimics, as the haptens for a conjugate vaccine. Therefore, the crucial LPS detoxification step is avoided. Following the synthesis of a large panel of SF2a O-SP fragments (21–26) and extensive investigation of their recognition by protective murine monoclonal IgG (mIgG) Abs, we showed that selected synthetic OSs induced an anti-O-SP Ab response in mice,

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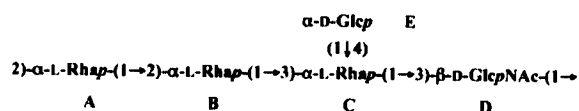
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<sup>4</sup> Abbreviations used in this paper: SF2a, *Shigella flexneri* 2a; i.n., intranasal; mIgG, monoclonal IgG; RU, repeating unit; OS, oligosaccharides; O-SP, O-specific polysaccharide; TT, tetanus toxoid.

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**FIGURE 1.** Basic structure of the repeating unit of the O-SP moiety of SF2a LPS.

when administered as tetanus toxoid (TT) conjugates. Increasing the length of the carbohydrate hapten from a penta- to a deca- and pentadecasaccharide representing one, two, and three biological RUs of SF2a O-SP (Fig. 1), respectively, improves the anti-LPS 2a IgG response (27). Here, we report on the protective capacity of those glycoconjugate-induced anti-LPS Abs against SF2a infection in mice, and show that the pentadecasaccharide [AB(E)CD]<sub>3</sub> is a functional mimic of the natural polysaccharide Ag. We also assess the different parameters influencing immunogenicity in mice. Finally, we present preclinical data demonstrating that [AB(E)CD]<sub>3</sub>-TT is a candidate for a synthetic carbohydrate-protein conjugate vaccine against SF2a infection.

## Materials and Methods

### Bacterial strains

*S. flexneri* 454, an invasive isolate of SF2a was the virulent strain of reference. For intranasal (i.n.) infection, bacteria were routinely grown on Luria-Bertani agar plates at 37°C, recovered from the plates, and diluted with 0.9% NaCl to an OD of 1 at 600 nm ( $5 \times 10^8$  CFU/ml). For counting of bacteria upon i.n. infection, lungs were recovered en bloc after cervical dislocation of mice and ground in 10 ml of sterile PBS (Ultra turrax T25 apparatus, IKA-Werke). Dilutions were then plated on trypticase soy broth plates for enumeration.

### Semi-synthetic glycoconjugates

The general synthetic and analytical protocols used to obtain the TT-conjugates have been previously described (27). Briefly, TT (12 mg, batch no. FA 045644, gift from Sanofi-Pasteur) was converted to maleimide-activated TT. Synthetic SF2a 5-acetylthioacetylated-penta- (28), deca-, and pentadecasaccharides (29), followed respectively by NH<sub>2</sub>OH and HCl (7.5 μl of a 2 M solution in 1 M potassium phosphate buffer, pH 6) were added to the activated TT (0.5 M in 0.1 M potassium PBS, pH 6) in molar ratios ranging from 1:5 to 1:15, and the mixtures were stirred for 2 h at room temperature. Following dialysis against 3 × 2 L of 0.05 M PBS, pH 7.4 at 4°C (MW cut-off 10,000 Da), and gel permeation chromatography on a Sepharose CL-6B column (1 m × 160 mm) in 0.05 M PBS, pH 7.4, the conjugates were stored at 4°C in the presence of thimerosal (0.01%). SF2a OS/TT molar ratio was assessed by SELDI-TOF MS (Supplementary material 1).<sup>5</sup>

### Biotinylated probes

Biotinylated penta-, deca-, and pentadecasaccharides were synthesized as described (27). The biotinylated linker mimics (Supplementary material 2)<sup>5</sup> were prepared accordingly. Thus, Biot-Mal-linker was synthesized in two steps, providing Biot-Mal-NHOH as side-product. Hydrolysis of EZ-link PEO-maleimide activated biotin (Pierce) provided Biot-Mal-OH.

### Immunogenicity studies

Seven-week old BALB/c mice were injected i.m. with AB(E)CD-TT, [AB(E)CD]<sub>2</sub>-TT, or [AB(E)CD]<sub>3</sub>-TT, three times at 3 wk intervals, followed by a fourth injection 1 mo after the third one, with the equivalent of 1, 2.5, or 10 μg of carbohydrate hapten, as indicated, per mouse and per injection, in the absence of adjuvant. The anti-LPS and anti-OS induced Ab responses were assessed 1 wk after the third and fourth injections by ELISA as previously described (27). Accordingly, to define the anti-linker Ab titers, biotinylated linker mimics (0.5 μg/well) were coated on plates previously incubated with avidin (1 μg/well, Sigma-Aldrich) for 1 h at 37°C. Anti-mouse IgG alkaline phosphatase-labeled conjugate (Sigma-Aldrich) was used as secondary Ab at a dilution of 1/5,000. For analysis of IgG subclasses, anti-mouse IgG1, IgG2a, IgG2b, and IgG3 alkaline phos-

phatase-labeled conjugates (Sigma-Aldrich) were used at a dilution of 1/5,000. For analysis of the cross-reactivity of mouse sera, plates were incubated with LPS purified from different *Shigella* strains as indicated. The Ab titer was defined as the last serum dilution given an OD of at least twice that of preimmune serum.

### Analysis of O-SP recognition by glycoconjugate-induced sera

Purified LPS 2a (2 μg/lane) was run in SDS-PAGE and silver-stained as previously described (30). Following Western blotting, nitrocellulose membranes were incubated with sera from mice immunized with [AB(E)CD]<sub>3</sub>-TT or whole killed SF2a bacteria as control. Sera were tested using a similar anti-LPS 2a Ab titer. Anti-mouse Ig HRP-labeled conjugate (Amersham Biosciences) at a dilution of 1/5000 was used as secondary Ab.

### Protective capacity of glycoconjugate-induced sera

To compare the protective capacity of anti-LPS 2a Abs induced upon immunization with AB(E)CD-TT, [AB(E)CD]<sub>2</sub>-TT, or [AB(E)CD]<sub>3</sub>-TT, mouse sera exhibiting a similar anti-LPS 2a Ab titer (1/12,800) were selected from each group of mice immunized with one of the conjugates. The selected immune sera (15 μl) were mixed with 10<sup>6</sup> CFU of virulent SF2a bacteria (5 μl) just before i.n. administration of the total 20 μl to naive mice. Measurement of lung-bacterial load (CFU/lungs) was done at 24 h postinfection. Two control groups were included: one receiving bacteria previously incubated with sera from mice immunized with B(E)CD-TT, but negative for the anti-LPS 2a Ab response, and one receiving bacteria previously incubated with preimmune serum. Three independent experiments including seven mice per group were run for each immune serum tested.

### Recognition of AB(E)CD, [AB(E)CD]<sub>2</sub>, and [AB(E)CD]<sub>3</sub> by sera of naturally infected individuals

ELISA was performed as described above using human sera from individuals naturally infected by SF2a against LPS 2a, biotinylated AB(E)CD, [AB(E)CD]<sub>2</sub>, or [AB(E)CD]<sub>3</sub> OSs as coating Ags. Anti-human IgG alkaline phosphatase-labeled conjugate (Sigma-Aldrich) was used as secondary Ab at a dilution of 1/2500. Eight paired sera obtained from Israeli soldiers before starting training cycles under field conditions and 1 to 2.5 mo after suffering from culture-proven SF2a shigellosis were tested.

### Statistical analysis

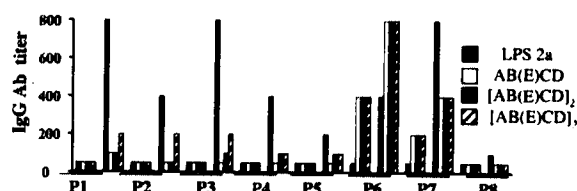
Significant differences were established using the Student's *t* test. Values of *p* < 0.05 were considered to be significant.

## Results

### Recognition of AB(E)CD, [AB(E)CD]<sub>2</sub>, and [AB(E)CD]<sub>3</sub> by sera of naturally infected individuals

Characterization of SF2a-specific determinants was performed using protective murine mIgGs (27). To assess whether the pentasaccharide AB(E)CD, the decasaccharide [AB(E)CD]<sub>2</sub>, and the pentadecasaccharide [AB(E)CD]<sub>3</sub> mimicking SF2a-specific determinants were the targets of the Ab response induced upon natural infection, ELISA was performed using a panel of human sera recovered from SF2a-naturally infected individuals and the corresponding preinfection sera as control. All infected patients elicited an anti-LPS 2a Ab response (Fig. 2). Except for patient P8, all the postinfection sera tested had an increased anti-OS Ab response as compared with preinfection ones. For patients P1, P2, and P3, the anti-[AB(E)CD]<sub>3</sub> Ab response was higher than those directed against AB(E)CD and [AB(E)CD]<sub>2</sub>, those were similar except for P3 exhibiting a slightly higher anti-[AB(E)CD]<sub>2</sub> Ab titer (Fig. 2). For patients P4 and P5, the anti-[AB(E)CD]<sub>2</sub> and anti-[AB(E)CD]<sub>3</sub> Ab responses were similar, and slightly higher than that measured for AB(E)CD. Finally, for patients P6 and P7, no difference was observed between the anti-OS Ab responses (Fig. 2). These data show that AB(E)CD, [AB(E)CD]<sub>2</sub>, and [AB(E)CD]<sub>3</sub> are targets of the Ab response elicited upon SF2a-natural infection.

<sup>5</sup> The online version of this article contains supplemental material.



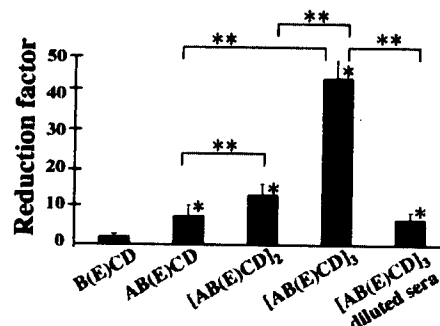
**FIGURE 2.** Synthetic haptens corresponding to 1, 2, and 3 SF2a O-SP RU are recognized by sera of SF2a-naturally infected patients. Sera from SF2a-naturally infected patients were tested in ELISA using biotinylated AB(E)CD, [AB(E)CD]<sub>2</sub>, [AB(E)CD]<sub>3</sub>, and purified SF2a LPS as coating Ags. IgG Ab titer against each Ag was measured. Eight paired sera (P1–P8), obtained from Israeli soldiers before starting training cycles under field conditions and 1 to 2.5 mo after suffering from culture proven SF2a shigellosis, were tested.

#### Induction of a protective anti-SF2a Ab response by AB(E)CD-TT, [AB(E)CD]<sub>2</sub>-TT and [AB(E)CD]<sub>3</sub>-TT

Selected AB(E)CD-TT, [AB(E)CD]<sub>2</sub>-TT, or [AB(E)CD]<sub>3</sub>-TT conjugates having an average carbohydrate to protein ratio of 14:1 based on colorimetric assay (31), were used to immunize mice four times at doses corresponding to 10  $\mu$ g of carbohydrate. To assess the protective efficacy of the anti-LPS 2a Abs induced upon immunization with those three conjugates, naive mice were passively i.n. administered with immune or preimmune sera previously incubated with virulent SF2a bacteria. Immune sera with the same anti-LPS 2a Ab titer, i.e., 1/12,800, were selected for each conjugate. It is noteworthy that the average of the anti-LPS 2a Ab titer induced by the three conjugates was previously shown to be 1/728, 1/5,200, and 1/26,000 for AB(E)CD-TT, [AB(E)CD]<sub>2</sub>-TT, and [AB(E)CD]<sub>3</sub>-TT, respectively (27). For AB(E)CD-TT and [AB(E)CD]<sub>2</sub>-TT, only 7 and 20% of mice, respectively, raised an anti-LPS 2a Ab titer of 1/12,800. In contrast, in 93% of mice immunized with [AB(E)CD]<sub>3</sub>-TT, the anti-LPS 2a Ab titer was equal or superior to 1/12,800 (with 65%, superior to 1/12,800). Protection was evaluated by measuring the CFU/lungs at 24 h postinfection and a reduction factor was calculated as the ratio between CFU/lungs in naive mice passively administered with pre-immune sera to that of naive mice passively administered with immune sera. Serum from mice immunized with B(E)CD-TT was used as a control because this conjugate does not induce any anti-LPS 2a Ab response (27). A significant but low level of protection was observed for AB(E)CD-TT-induced Abs with only a 7-fold reduction in the bacterial load as compared with control group. The level of protection was significantly higher with [AB(E)CD]<sub>2</sub>-TT-induced Abs as compared with AB(E)CD-TT ( $p = 0.0023$ ), but still only a 13-fold reduction in the bacterial load was measured. The best protection, i.e., a 45-fold reduction, was obtained with sera from mice immunized with [AB(E)CD]<sub>3</sub>-TT. The reduction factor was significantly higher than that measured with sera from mice immunized with AB(E)CD-TT ( $p = 1.8 \times 10^{-6}$ ) and [AB(E)CD]<sub>2</sub>-TT ( $p = 1.06 \times 10^{-5}$ ), respectively (Fig. 3). Protection conferred with sera from mice immunized with [AB(E)CD]<sub>3</sub>-TT was shown to be dose-dependant because a 100 time-dilution of [AB(E)CD]<sub>3</sub>-TT-induced sera led to a 10-fold decrease of the reduction factor. These results demonstrate that, among the OSs tested, [AB(E)CD]<sub>3</sub> is the best functional mimic of the SF2a O-SP. Detailed analysis of parameters influencing the immunogenicity were then focused on [AB(E)CD]<sub>3</sub>-TT glycoconjugate.

#### Recognition of SF2a O-SP chains by sera of mice immunized with [AB(E)CD]<sub>3</sub>-TT

LPS is associated to a ladder type profile derived from the heterogeneity in the number of RUs per O-SP chain when run in Tricine

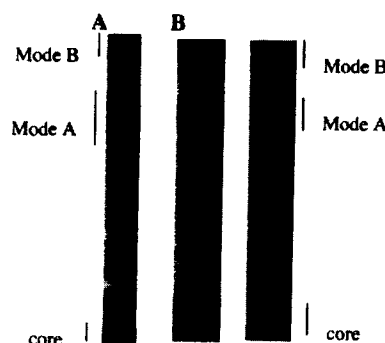


**FIGURE 3.** [AB(E)CD]<sub>3</sub>-TT induces the best protective anti-LPS 2a Ab response. Mouse sera exhibiting a similar anti-LPS 2a Ab titer (1/12,800) were selected from each group of mice immunized with one of the conjugates. The selected immune sera were mixed with  $10^6$  CFU of virulent SF2a bacteria before i.n. administration to naive mice. Measurement of CFU/lungs was done 24 h postinfection. Two control groups were included: one receiving bacteria previously incubated with sera from mice immunized with B(E)CD-TT, but negative for the anti-LPS 2a Ab response, and one receiving bacteria previously incubated with preimmune serum. Three independent experiments including seven mice per group were run for each immune serum tested. \*,  $p$  value < 0.05 (Student's  $t$  test) when comparing sera from mice immunized with AB(E)CD-, [AB(E)CD]<sub>2</sub>-, and [AB(E)CD]<sub>3</sub>-TT conjugates to that of mice immunized with B(E)CD-TT. \*\*,  $p$  value < 0.05 (Student's  $t$  test) when comparing the conjugates, as indicated.

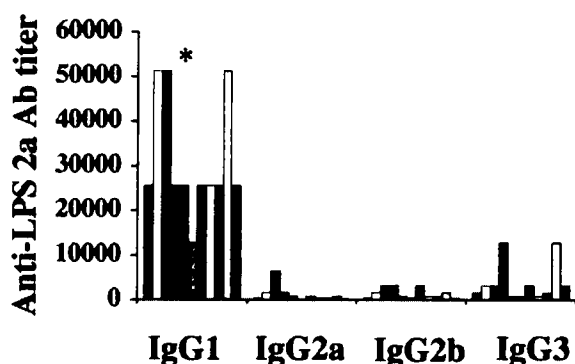
SDS-PAGE. For SF5a, for example, two major LPS populations coexist, with an average modal number of RUs of  $\sim 15$  (mode A) and 100 (mode B) (30). We investigated the length of LPS 2a chains recognized by [AB(E)CD]<sub>3</sub>-TT-induced Abs and whole-killed SF2a bacteria induced Abs. As observed in Fig. 4A, two major modal forms, A and B, were present for SF2a LPS. Upon immunoblotting, except for core recognition, modes A and B SF2a LPS were both recognized by sera from mice immunized with whole-killed SF2a bacteria (Fig. 4B, left panel) or [AB(E)CD]<sub>3</sub>-TT (Fig. 4B, right panel). These results demonstrating the recognition of long O-SP chains by [AB(E)CD]<sub>3</sub>-TT induced-IgG Abs at least partially account for the functional mimicry of O-SP by [AB(E)CD]<sub>3</sub>.

#### Anti-LPS 2a IgG subclasses induced by [AB(E)CD]<sub>3</sub>-TT

Analysis of the anti-LPS 2a IgG subclasses induced upon immunization of mice with [AB(E)CD]<sub>3</sub>-TT was performed by ELISA. For each mouse, the anti-LPS 2a IgG1 response was significantly predominant over that elicited for IgG2a, 2b, and



**FIGURE 4.** Analysis of O-SP recognition by [AB(E)CD]<sub>3</sub>-TT-induced sera. Purified LPS 2a was run in SDS-PAGE, silver-stained (A) or Western blotted (B) and incubated with sera from mice immunized with whole killed SF2a bacteria (B, left panel) or [AB(E)CD]<sub>3</sub>-TT (B, right panel).



**FIGURE 5.** [AB(E)CD]<sub>3</sub>-TT predominantly induces an anti-LPS 2a IgG1 response. IgG subclasses were analyzed by ELISA using [AB(E)CD]<sub>3</sub>-TT-induced sera and LPS 2a as coating Ag. Individual response toward IgG1, IgG2a, IgG2b, and IgG3 is shown for a group of 11 immunized mice representative of ~40 mice that have been immunized with [AB(E)CD]<sub>3</sub>-TT in different experiments. \*, *p* value < 0.05 (Student's *t* test) when comparing the anti-LPS 2a IgG1 Ab titer to that of the other IgG subclasses.

3 (*p* values =  $1.9 \times 10^{-8}$ ,  $1.9 \times 10^{-8}$ , and  $1.8 \times 10^{-7}$ , respectively) (Fig. 5). No significant difference was observed between the anti-LPS 2a IgG2a, 2b, and 3 Ab responses, with low Ab titers induced except for two of 11 mice that exhibited a marked IgG3 Ab response. These results show that IgG1 is the IgG subclass mainly induced upon immunization of mice with [AB(E)CD]<sub>3</sub>-TT. It is worth to mention that 2 mo after the boost, the anti-LPS 2a IgG response was only slightly lowered (data not shown).

#### Cross-reactivity of sera induced by [AB(E)CD]<sub>3</sub>-TT toward other prevalent *S. flexneri* LPS

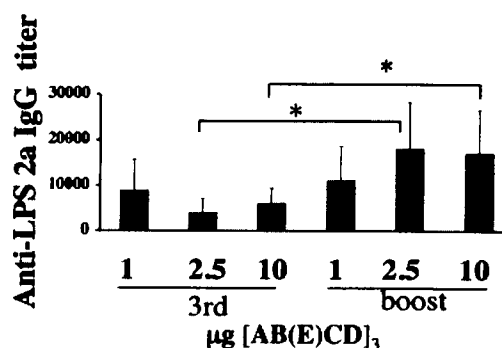
To assess the serotype-specificity of the anti-LPS Abs induced by [AB(E)CD]<sub>3</sub>-TT, ELISA was performed using sera of [AB(E)CD]<sub>3</sub>-TT-immunized mice and purified LPS from other *S. flexneri* serotypes as coating Ags. No Ab response against LPS 1b or LPS 3a, two other prevalent *S. flexneri* strains (12), was detected. The study was therefore extended to other *S. flexneri* serotypes. No cross-reactivity was detected using LPS purified from SF2b, 3b, 5b, and X strains, whereas SF5a and Y LPS were slightly recognized. Altogether, these results indicate that the Ab response elicited upon immunization of mice with [AB(E)CD]<sub>3</sub>-TT is highly specific for SF2a.

#### Influence of the dose on the immunogenicity of [AB(E)CD]<sub>3</sub>-TT

Preliminary immunogenicity data with [AB(E)CD]<sub>3</sub>-TT were obtained using 10 µg of [AB(E)CD]<sub>3</sub> per mouse and per dose (27). Following the same immunization protocol, immunogenicity of [AB(E)CD]<sub>3</sub>-TT was assessed using amounts of glycoconjugates corresponding to 1 and 2.5 µg of [AB(E)CD]<sub>3</sub> per mouse and per dose. The anti-LPS 2a Ab titers, raised with these lower amounts of carbohydrate, were not significantly different from that induced with 10 µg of hapten, both after the third dose and the boost (Fig. 6), demonstrating that for [AB(E)CD]<sub>3</sub>-TT, 1 µg of [AB(E)CD]<sub>3</sub> is an efficient immunizing dose in mice.

#### Influence of carbohydrate hapten loading on the immunogenicity of [AB(E)CD]<sub>3</sub>-TT

As stated above, [AB(E)CD]<sub>3</sub>-TT conjugates used for previous studies (27), and for those presented here, had an average ratio of 14:1 OS chains/protein. To assess the influence of [AB(E)CD]<sub>3</sub> loading on immunogenicity, new sets of [AB(E)CD]<sub>3</sub>-TT con-

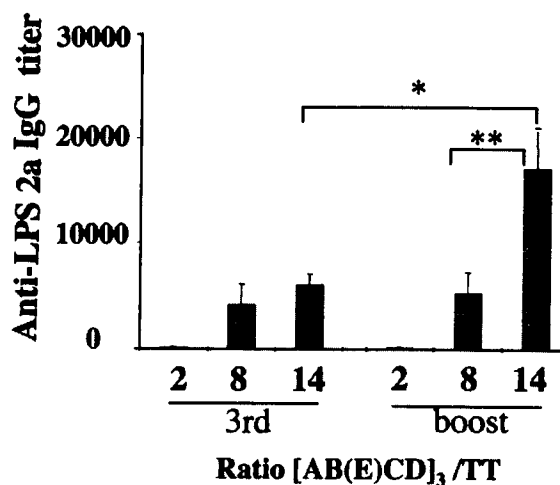


**FIGURE 6.** Influence of the immunizing dose on [AB(E)CD]<sub>3</sub>-TT immunogenicity. Anti-LPS 2a Ab titer was measured by ELISA in sera from mice immunized with [AB(E)CD]<sub>3</sub>-TT using an equivalent of 1, 2.5, or 10 µg of carbohydrate hapten.

jugates bearing an average hapten molar loading of two, eight, and 14 were synthesized and evaluated by immunizing mice with an equivalent of 10 µg of OS per mouse and per dose. The [AB(E)CD]<sub>3</sub>-TT conjugate with a ratio of two induced no anti-OS Abs (data not shown), and consequently no anti-LPS 2a Abs (Fig. 7). For a molar ratio of eight, a similar anti-LPS 2a Ab response was observed after the third immunization and the boost (*p* = 0.002), in contrast to ratio 14 for which a significant increase was observed after the boost (*p* = 0.008), giving rise to the best response observed so far. These results demonstrate that an average [AB(E)CD]<sub>3</sub>/TT ratio of 14 provided the best immunogen of all those tested.

#### Analysis of the anti-linker Ab response induced by [AB(E)CD]<sub>3</sub>-TT

Construction of the [AB(E)CD]<sub>3</sub>-TT conjugates relies on the maleimide-thiol ligation chemistry, selected for its high chemoselectivity and high-yielding coupling efficacy at controlled pH. The *N*-(γ-maleimidobutyryloxy) amide linker used herein was selected based on the finding that use of the more flexible alkyl-type maleimide spacers could generally overcome the immunogenicity of



**FIGURE 7.** Influence of carbohydrate hapten loading on [AB(E)CD]<sub>3</sub>-TT immunogenicity. Anti-LPS 2a Ab titer was measured by ELISA in sera from mice immunized with [AB(E)CD]<sub>3</sub>-TT displaying an average carbohydrate/TT ratio of 2, 8, and 14. \*, *p* value < 0.05 (Student's *t* test) when comparing for a given dose the Ab titer induced after the third immunization and the boost. \*\*, *p* value < 0.05 (Student's *t* test) when comparing the three doses after the third immunization or the boost.

cyclic maleimide linkers in animal models (32, 33). However, linear maleimide linkers may also be immunogenic (6, 34), checking whether it was the case with our [AB(E)CD]<sub>3</sub>-TT conjugate was therefore mandatory. To investigate the anti-linker Ab response, the corresponding Biot-Mal-linker derivative, bearing the whole 13 atom-long spacer present in [AB(E)CD]<sub>3</sub>-TT conjugates, was synthesized. Side-products derived from hydroxylamine addition onto maleimide (Biot-Mal-NHOH) or maleimide hydrolysis (Biot-Mal-OH) were also isolated because any side-product could be identified as a potential source of neo-epitopes (33). Independently of the OS molar loading onto the protein, or of the amount of carbohydrate used per mouse per dose, no anti-linker IgG Ab was detected against any of the three newly synthesized biotinylated probes used as coating Ags (data not shown). These data indicate that upon immunization with [AB(E)CD]<sub>3</sub>-TT, there is no detectable Ab response induced against the *N*-( $\gamma$ -maleimidobutyryloxy) amide linker or side-products thereof. Interestingly, the influence of the linker on immunogenicity may highly depend on the nature and intrinsic immunogenicity of the carbohydrate hapten. Along this line, we showed that neither [AB(E)CD]<sub>2</sub>-TT nor [AB(E)CD]<sub>1</sub>-TT gave rise to detectable anti-linker Abs.

## Discussion

In the past decades, interest in synthetic microbial carbohydrate-based vaccines, thus termed third generation polysaccharide vaccines, has emerged as one among the many exploding fields of carbohydrate medical applications. To our knowledge, only one such candidate vaccine has yet gone through clinical trials in the case of bacterial infections (8). The resulting licensing of Quimi Hib in Cuba in 2003 demonstrates the potential of the strategy in humans. However, despite an increasing number of encouraging reports, efficient third generation bacterial polysaccharide vaccines remain at an early stage of development (7, 9, 10). By demonstrating the induction of a protective anti-LPS Ab response against SF2a, using a conjugate incorporating a rationally designed synthetic OS mimic of LPS 2a, the current study provides an additional example of the potential of such an approach.

There is yet no established rule to define the appropriate hapten length resulting in optimal functional mimicry of the native Ag that would prevent from performing immunogenicity/protection studies. Thus, performing those studies, we showed that [AB(E)CD]<sub>3</sub> is the best mimic among the studied haptens. Indeed, [AB(E)CD]<sub>3</sub>-TT induced sera confer the best protection in mice, and we previously reported that [AB(E)CD]<sub>3</sub>-TT is the only conjugate among all those tested that induces an anti-LPS 2a immune response in 100% mice (27). It is noteworthy that parameters such as antigenicity measured using mouse mIgGs specific for SF2a serotype and human sera, as well as structural data, would not have favored the selection of [AB(E)CD]<sub>3</sub>. Indeed, inhibition ELISA analysis showed either a similar recognition or a loss in recognition upon elongation of [AB(E)CD]<sub>2</sub> by one RU, depending on the mIgGs (Supplementary material 3).<sup>5</sup> Besides, AB(E)CD, [AB(E)CD]<sub>2</sub>, and [AB(E)CD]<sub>3</sub> were similarly recognized by human sera from patients naturally infected with SF2a, demonstrating that O-Ag epitopes recognized by human sera are, to some extent, present in haptens corresponding to a small number of RUs. In addition, available structural data on [AB(E)CD]<sub>2</sub> and [AB(E)CD]<sub>3</sub>, in complex with one of the available protective mAbs, mIgG F22-4, demonstrated that the nine residue-epitopes that bind to mIgG F22-4 Fab are already present in the decasaccharide (35). As an opening to challenge the topic, additional structural and physicochemical investigations are in progress to unravel the molecular basis for accurate mimicking of SF2a O-SP by [AB(E)CD]<sub>3</sub>.

Regarding the protective capacity of [AB(E)CD]<sub>3</sub>-TT induced sera, in contrast to reports showing that synthetic haptens corresponding to only one RU or less could provide Ab-mediated protection in animal models (36–38), at least two RUs were needed for SF2a, with three RUs giving rise to the best protection observed so far. Those data fit several observations indicating that a minimum of two contiguous RUs are required to induce anti-polysaccharide Abs in the case of heteropolysaccharide Ags (39, 40), although this is not always the case (38). Besides, the increased protective capacity of [AB(E)CD]<sub>3</sub>-TT over [AB(E)CD]<sub>2</sub>-TT parallels data reported for *H. influenzae* b (41), and the corresponding increased immunogenicity supports data described for *S. dysenteriae* 1 (40). Alternative options toward additional increase of the protective anti-LPS 2a Ab response will be investigated (42). Indeed, in direct support to our observations, protection against shigellosis is thought to be correlated to the level of anti-LPS Abs (17, 18), although the threshold of the anti-LPS Ab response required to ensure protection is yet unknown. To further detail the protective anti-LPS Ab response induced by [AB(E)CD]<sub>3</sub>-TT, we observed that three immunizations were required to promote an optimal anti-LPS IgG response. An additional boost only slightly increased the anti-LPS IgG titer by a factor of two to three. In full agreement with a T-dependent immune response, an anti-LPS IgM titer was detected after the first immunization only (data not shown).

Numerous factors, including the polysaccharide itself, influence the pattern of IgG subclass response (18). Analysis of the anti-LPS IgG subclasses revealed that [AB(E)CD]<sub>3</sub>-TT induces in mice a Th2-type immune response mainly mediated by IgG1. Interestingly, this particular profile mimics the Th2-type response observed in humans following natural SF2a infection (18) or immunization with a detoxified SF2a LPS-protein conjugate (43). Although serotyping analysis has demonstrated that *S. flexneri* O-SPs share several epitopes (15), data on the field suggest that protection induced by natural infection is serotype-specific. Immunization in experimental models with vaccine candidates was only occasionally shown to induce cross-protection (44, 45). Herein, we show that [AB(E)CD]<sub>3</sub>-TT induced sera do not significantly cross-react with serotypes 1b, 2a, 3a, 5a, 5b, X, and Y LPSs. Overall, these data suggest that [AB(E)CD]<sub>3</sub>-TT induces in mice an Ab response that is highly SF2a-oriented, as observed in naturally infected patients.

Shortening the hapten length also impacts on the number of exposed carbohydrate epitopes, and consequently on the carrier and linker relative visibility by the host's immune system, possibly leading to divergence of the Ab response. For example, in the case of a hapten possessing a low inherent immunogenicity, induction of anti-linker Abs was reported to be detrimental to induction of anti-hapten Abs (33, 34). Along this line, it is noteworthy that no Ab response was induced against the maleimide linker used for conjugation of [AB(E)CD]<sub>3</sub> to TT. As additional support to this finding, the same observation remained true if shorter haptens, one and two RUs, respectively, were involved (data not shown). Interestingly, glycoconjugates prepared using maleimide linkers were occasionally administered in humans with no reported negative influence on hapten-immunogenicity (8, 46). Furthermore, the routine administration of Quimi Hib to infants (8) suggests that a maleimide linker is indeed appropriate for use in humans.

In the present stage of development of synthetic carbohydrate-protein conjugate vaccines, additional investigation is required to better understand the impact of interdependent parameters on immunogenicity and protective efficacy. Having selected TT, a medically acceptable carrier for use in human, efforts were put on analyzing the amount per dose and molar loading. There is no

established correlation between hapten loading and protective efficacy (37, 47, 48). Here, we clearly show that it is a critical parameter. The [AB(E)CD]<sub>3</sub>-TT conjugate with a hapten loading of two was the less potent one, failing to induce an anti-LPS or anti-[AB(E)CD]<sub>3</sub> IgG response when administered three times at 10 µg carbohydrate/dose. Interestingly, after the third immunization there was no significant difference between the immunogenicity of the [AB(E)CD]<sub>3</sub>-TT conjugates displaying a hapten molar loading of 8 and 14, respectively, suggesting that this range of hapten molar loading induces appropriate B cell receptor cross-linking, without masking important Th epitopes. However, despite an increased immunogenicity observed after the boost for the [AB(E)CD]<sub>3</sub>-TT conjugate having a 14:1 OS chains/protein ratio, molar loading resulting in maximal immunogenicity remains yet unknown. Additional improvement may therefore be in reach, as illustrated in an elegant study on *S. dysenteriae* type 1 where the hapten loading was increased to a point resulting in a decrease in immunogenicity (40). Identifying the highest reproducible hapten molar loading inducing the highest anti-LPS Ab response is important to the issue of carrier overloading (49). Indeed, using glycoconjugates of similar potency but increased hapten loading would result in lowering the amount of carrier for a given administered amount of carbohydrate.

Regarding the influence of the dose, for the most recently licensed polysaccharide conjugate vaccines, for example Prevnar, the amount of polysaccharide per dose varies from 2 to 4 µg in average, depending on the polysaccharide chemical structure. Analogously, lowering the amount of immunogen administered to animal models is the general tendency. Along this line, our data showing that when used at doses corresponding to 2.5 or 1 µg of synthetic carbohydrate, [AB(E)CD]<sub>3</sub>-TT induces high anti-LPS IgG titers in mice are consistent with most recent investigations on synthetic OS-based conjugate vaccines (37, 40). To our knowledge, reports on immunization with 1 µg carbohydrate amounts or less are scarce but support our findings (37, 41). For example, a synthetic tetrasaccharide-CRM<sub>197</sub> type 3 pneumococcal conjugate was shown to induce anti-polysaccharide Ab titers which were high enough to confer protective immunity against intraperitoneal challenge with a lethal dose of *S. pneumoniae* type 3 when used in mice at doses corresponding to 0.25 µg of carbohydrate (37). It is noteworthy that data suggest that synthetic OS-based conjugate vaccines are equally or even more strongly immunogenic in comparison to their native polysaccharide conjugate counterpart (8, 40).

The poor accessibility of well-defined complex oligosaccharides has for long impaired investigations on the potential of synthetic OS-based conjugate vaccines for medical applications. This is no longer the case, as demonstrated by the number of reports (10) and the recent breakthrough in the field of *Haemophilus influenzae* b glycoconjugate vaccines (8). Along this line, the pentadecasaccharide [AB(E)CD]<sub>3</sub> was initially synthesized in 44 chemical steps following a convergent approach based on three building blocks (29). The synthesis was thought compatible with scale-up. Nevertheless, additional validation of the whole strategy was, in our opinion, a prerequisite. In the course of this study, we could fully reproduce and even improve the reported synthesis. The pentadecasaccharide is now obtained in 40 steps, which include several improvements (T. H. Kim and L. A. Mulard, unpublished results).

In conclusion, considering the drawbacks of orally administered, live attenuated *Shigella* vaccine candidates (14), and the absence of published data on the efficacy of *Shigella*-detoxified LPS-conjugate vaccines in young children living in endemic areas, the current study emphasizes the potential of selected synthetic oligosaccharides as immunogenic mimics of the bacterial surface

polysaccharide. The proof of concept in humans is needed urgently to definitely establish the development of such rationally designed synthetic carbohydrate-based conjugates as a promising alternative strategy for *Shigella* vaccines.

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## Disclosures

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